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Mechanisms of Extrahepatic Tumor Induction by Peroxisome Proliferators in Male CD Rats

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Wyeth-14,643 (WY) and ammonium perfluorooctanoate (C8) belong to a diverse class of compounds which have been shown to produce hepatic peroxisome proliferation in rodents. From previous work, WY, but not C8, has been shown to produce hepatocellular carcinoma in rats, while C8 has been shown to produce Leydig cell adenomas. In addition, based on a review of bioassay data a relationship appears to exist between peroxisome-proliferating compounds and Leydig cell adenoma and pancreatic acinar cell hyperplasia/adenocarinoma formation. To further investigate the relationship between peroxisome-proliferating compounds and hepatic, Leydig cell, and pancreatic acinar cell tumorigenesis, a 2-year feeding study in male CD rats was initiated to test the hypothesis that peroxisome proliferating compounds induce a tumor triad (liver, Leydig cell, pancreatic acinar cell), and to examine the potential mechanism for the Leydig cell tumors. The study was conducted using 50 ppm WY and 300 ppm C8. The concentration of WY in the diet was decreased to 25 ppm on test day 301 due to increased mortality. In addition to the *ad libitum* control, a second control was pair-fed to the C8 group. Interim sacrifices were performed at 1- or 3-month intervals. Peroxisome proliferation measured by β-oxidation activity and cell proliferation were measured in the liver and testis at all time points and in the pancreas beginning at the 9-month time point (cell proliferation only). Serum hormone concentrations (estradiol, testosterone, LH, FSH, and prolactin) were also measured at each time point. Increased relative liver weights and hepatic β-oxidation activity were observed in both the WY- and C8-treated rats at all time points. In contrast, hepatic cell proliferation was significantly increased only in the WY-treated group. Neither WY nor C8 significantly altered the rate of Leydig cell β-oxidation or Leydig cell proliferation when compared to the control groups. Moreover, the basal rate of β-oxidation in Leydig cells was approximately 20 times less than the rate of hepatic β-oxidation. There were no biologically meaningful differences in serum testosterone, FSH, prolactin, or LH concentrations in the WY- and C8-treated rats when compared to their respective controls. There were, however, significant increases in serum estradiol concentrations in the WY- and C8-treated rats at 1, 3, 6, 9, 15, 18, and 21 months. At 12 months, only the C8-treated rats had elevated serum estradiol concentrations when compared to the pair-fed control. Histopathological evaluation revealed compound-related increases in liver, Leydig cell, and pancreatic acinar cell tumors in both WY- and C8-treated rats. The data support the hypothesis that the peroxisome-proliferating compounds induce the previously described tumor triad. In addition, both C8 and WY produced a sustained increase in serum estradiol concentrations that correlated with the potency of the 2 compounds to induce Leydig cell tumors (i.e., WY caused a more consistent sustained increase in serum estradiol throughout the entire study, and more specifically at the end of the study, than did C8). This study suggests that estradiol may play a role in enhancement of Leydig cell tumors in the rat, and that peroxisome proliferators may induce tumors via a non-LH type mechanism.

Key Words: **peroxisome proliferators; estradiol; Leydig cell.**

A large number of structurally and chemically diverse compounds have been shown to cause peroxisome proliferation, induction of peroxisomal enzymes, and hepatocellular carcinoma. Based on a review of bioassay data for non-Fischer 344 (F344) strains of rat, a relationship also appears to exist between compounds which produce peroxisome proliferation and Leydig cell adenoma formation. Leydig cells, which are found within the testis, are the main site of testosterone biosynthesis. By 2 years of age, the incidence of spontaneous Leydig cell tumors in the F344 rat approaches 100%, which precludes detection of chemically-induced Leydig cell tumors in this strain (Lang, 199; Turek and Desjardins, 1979). However, several known peroxisome proliferators have been shown to induce Leydig cell tumors in non-F344 strains of rat: clofibrate (Tucker and Orton, 1995), gemfibrozil (Fitzgerald *et al.*, 1981), HCFC-123 (Malley *et al.*, 1995), methylclofenapate (Tucker and Orton, 1995), perchloroethylene (Mennear, 1986), and trichloroethylene (TCE) (Maltoni *et al.*, 1988; Mennear, 1988). These data suggest that it is possible that many if not all peroxisome proliferators could produce Leydig cell tumors if tested in a strain of rat other than the F344.

An initial hypothesis for the mechanism of induction of Leydig cell tumors was that there was an increase in peroxisomes, and the tumor induction occurred in a manner similar to that of the liver. However in a series of short-term studies,

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which used both electron microscopy and biochemical methods, it was found that C8 and WY do not induce peroxisome production in the Leydig cells (Biegel *et al.,* 1992), although peroxisomes are present in this cell type. Additionally, C8 was found to decrease testosterone and increase estradiol concentrations *in vivo* and directly inhibit testosterone production when incubated with isolated Leydig cells (Biegel *et al.*, 1995). Several other peroxisome proliferators have also been shown to inhibit testosterone production using isolated Leydig cells (Liu *et al.*, 1996a). Therefore, it appears that Leydig cell tumors are not due to an increase in peroxisomes, but may be due to a disruption of the hypothalamic-pituitary-testicular (HPT) axis. To further investigate the relationship between peroxisome-proliferating compounds and hepatic and Leydig cell tumorigenesis, a 2-year feeding study was initiated using Wyeth-14,643 (WY) and ammonium perfluorooctanoate (C8) to test the hypothesis that peroxisome-proliferating compounds induce a tumor triad (liver, Leydig cell, pancreatic acinar cell) and to examine the potential mechanism for the Leydig cell tumors. The CD rat was selected because it has a low spontaneous incidence of Leydig cell tumors (~5%) (Cook *et al.*, 1999; Lang, 1992). C8 was selected because it has been shown to produce Leydig cell adenomas and also induce peroxisome proliferation. WY was selected as a model for the class of compounds known to be peroxisome proliferators, and it is a potent inducer of hepatic peroxisomes and hepatocellular carcinoma (Marsman *et al.*, 1988). WY has not been reported to produce Leydig cell tumors; however, all the bioassay studies conducted to date have used the F344 strain of rat. Therefore, this study will determine whether exposure to WY will produce Leydig cell tumors in a CD rat at a dietary concentration that produces liver tumors. Six months into this study, hydrochlorofluorocarbon 123 (HCFC-123), a known peroxisome proliferator, was shown to produce pancreatic acinar cell tumors (Malley *et al.*, 1995), this finding prompted the addition of the pancreas as an endpoint in this mechanistic bioassay.

MATERIALS AND METHODS

Test material, diet preparation, and analyses. C8 (98–100% pure) was supplied by the Polymer Products Department (DuPont, Wilmington, DE). Wyeth-14,643 (WY) was purchased from Chemsyn Science Laboratories (Lenexa, KS). The stability of C8 and WY were confirmed by analyses near the beginning, middle, and end of the study. At the beginning of the study and at the 3-, 6-, 12-, 18-, and 24-month time points, samples were collected to verify the concentration of test compounds in the diets. These samples were stored frozen (−20°C) until analyzed. At all time points, the concentration was within 10% of the nominal concentration.

C8 and WY were added to PMI® Feeds, Inc. Certified Rodent Diet #5002 (St Louis, MO) and thoroughly mixed for approximately 6 min in a high-speed Hobart mixer to assure homogeneous distribution in the diet. Analyses of the diets determined that the test compounds were homogeneously distributed. During the test period, rats in each group were fed, *ad libitum*, a diet of PMI® Feeds, Inc. Certified Rodent Diet #5002, which contained 0, 300 ppm C8, or 50 ppm WY. The concentration of WY was decreased to 25 ppm on test day 301, due to increased mortality. As a result, no WY-treated rats were sacrificed for biochemical or pathological evaluation at the 15-month time point.

Test species. Twenty-one day old male Crl:CD® BR (CD) rats were purchased from Charles River Breeding Laboratories (Raleigh, NC). Upon receipt, rats were placed in stainless steel, wire mesh cages, individually housed, and quarantined for 3 weeks. The rats were released from quarantine by the laboratory veterinarian and selected for the study on the bases of body weights and freedom from clinical signs of disease or injury during the quarantine period. Rats were then divided by computerized, stratified randomization into treatment groups so that there were no statistically significant differences among group body weight means. Rats were assigned to the *ad libitum* control group (control), control pair-fed rats to the C8 group (CP-C8), the 300-ppm C8 group, or the 50-ppm WY group. After assignment to treatment groups (n = 156/group), each rat was assigned a unique 6-digit number, and designated for either hormonal evaluation (10/group/time point), cell proliferation evaluation (6/group/time point), or evaluation of peroxisome proliferation (6/group/time point). Animal rooms were maintained at a temperature of 23 ± 1°C, a relative humidity of 50 ± 10%, and were artificially illuminated (fluorescent light) on a 12-h light/dark cycle (approximately 0600–1800 hours). In a few instances, the temperature/humidity were outside the acceptable ranges, but the magnitude/duration were minimal and judged to be of no consequence. All rats were provided tap water and PMI® Feeds, Inc. Certified Rodent Diet #5002, *ad libitum*. All rats were approximately 49 days of age on the day of study start.

All rats were housed individually in stainless steel, wire-mesh cages during the test period. Cage-side examinations were conducted at least once daily throughout the study. At each weighing, rats were individually handled and carefully examined for abnormal behavior and/or appearance. Rats were weighed once a week during the first 3 months and once every other week for the remainder of the study. Rats pair-fed to the C8 group had food consumption determined twice per week for the first 3 weeks. The CP-C8 group then received the same amount of food consumed by the C8-treated rats in the previous food consumption or weighing interval. Feed jars containing the mean daily food consumption were replaced daily. After the first 3 weeks, the amount of food consumed by each test group was determined weekly and, after 3 months, every 2 weeks. From these determinations and mean body weight data, mean daily food consumption, mean food efficiency, and intake of the test compounds were calculated.

Hormonal measurements. Ten rats from each group were randomly selected at each sampling time point for hormonal analysis. Blood was collected from the tail vein approximately 1, 3, 6, 9, 12, 15, 18, and 21 months after initiation of the study. For blood collection, rats were restrained using Narco Bio-Systems (Houston, TX) heated restrainers and blood was collected without anesthesia. Serum was prepared and frozen between −65 and −85°C until analyzed for testosterone, estradiol, luteinizing hormone (LH), follicle stimulating hormone (FSH), and prolactin concentrations. At each sampling time point, all serum samples were analyzed simultaneously in duplicate, using the same lot number kit for each of the designated hormones, in order to reduce variability. Testosterone (catalog #TKTT5) and estradiol (catalog #KE2D5) concentrations were determined using radioimmunoassay kits from Diagnostic Products Corp. (Los Angeles, CA). FSH (catalog #RPA.550), LH (catalog #RPA.552), and prolactin (catalog #RPA.553) concentrations were determined using radioimmunoassay kits from Amersham Corp. (Arlington Heights, IL).

Pathological evaluation. Rats were euthanized at interim time points 1, 3, 6, 9, 12, 15, 18, and 21 months. At each time point, 6 rats/group were selected for evaluations of cell proliferation and 6/group for peroxisome proliferation. Rats were euthanized by chloroform anesthesia and exsanguination. Testes, epididymides, accessory sex gland (ASG) unit with fluid, coagulating gland/seminal vesicle with fluid removed, prostate, and liver were weighed. Immediately after weighing, the liver and testes from animals selected for peroxisome proliferation evaluation were placed in ice-cold homogenization buffer for peroxisomal preparation. The following tissues were collected from rats selected for cell proliferation evaluation: testes, epididymides, ASG, liver, duodenum, pituitary, and all organs with gross lesions.

All rats surviving the 24-month test period were euthanized by chloroform anesthesia and exsanguination and were necropsied. Brain, heart, liver, spleen,

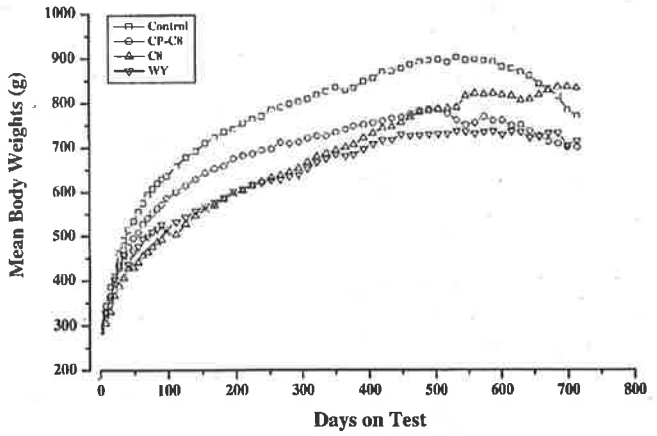

FIG. 1. Effect of C8 and WY on body weights of male rats over the course of the 2-year feeding study. Mean body weights were decreased in male rats fed 300 ppm C8 and 25/50 ppm WY.

kidneys, ASG unit, coagulating gland/seminal vesicles with fluid removed, prostate, epididymides, and testes were weighed at necropsy. The liver, testes, epididymides, pancreas, and organs with gross lesions were examined microscopically; single sections were examined on H & E stained slides. The morphologic criteria for diagnosis of proliferative pancreatic lesions were based on the recommendations of Hansen and co-workers (1995), which defines a proliferative acinar lesion as an adenoma if the diameter is greater than or equal to 5 mm. A Leydig cell adenoma was defined as a lesion with a diameter greater than 3 tubules.

Cell proliferation evaluation. Six days prior to euthanization at each of the time points, animals designated for cell proliferation evaluation were anesthetized by an injection of ketamine and xylazine, and Alzet® osmotic pumps (Palo Alta, CA) containing 20 mg/ml 5-bromo-2′-deoxyuridine (BrdU) dissolved in 0.5 N sodium bicarbonate buffer were implanted subcutaneously. At sacrifice, tissues were collected and fixed for cell proliferation analysis. The labeling index was determined for hepatocytes and Leydig cells at each of the specified time points. Additionally, the pancreas was collected at the 9-, 12-, 15-, 18-, and 21-month time points and labeling indices for pancreatic acinar cells were determined. The duodenum was used as a positive control for staining of labeled cells. For each tissue type, one thousand cells were scored.

Peroxisomal preparation. β-Oxidation activity from the liver and Leydig cell peroxisomes was measured at all of the interim time points from rats designated for evaluation of peroxisome proliferation. The livers were homogenized (1 g tissue/4 ml buffer) in homogenization buffer (0.1 M potassium phosphate buffer at pH 7.4, containing 0.25 mM sucrose, 1.0 mM EDTA, 2.0 mM glutathione, 4.0 mM magnesium chloride, and 50 μM leupeptin) with a polytron. The testes were decapsulated, digested with collagenase, and Leydig cells were isolated from Percoll gradients according to the method of Biegel and co-workers (1992). The Leydig cells were resuspended in homogenization buffer and homogenized with a polytron. The liver and Leydig cell homogenates were centrifuged at 600 × *g* for 15 min at 2°C. The 600 × *g* supernatant was removed and centrifuged at 15,000 × *g* for 15 min at 2°C. The 15,000 × *g* pellet was resuspended in a final volume of 4.0 ml homogenization buffer, aliquoted, and stored between −65 and −85°C until analyzed for β-oxidation activity. The protein concentration of the peroxisomal fractions was determined using Bio-Rad protein assay dye and BSA as a standard (Bradford, 1976).

Peroxisomal β-oxidation evaluation. β-oxidation activity, a quantitative measurement of peroxisome proliferation, was determined using the method of Lazarow (1981). Briefly, the cyanide-insensitive β-oxidation activity was measured using 5 μg hepatic peroxisomal protein/tube (0.5 mg protein/ml) and incubated at 37°C for 10 min with [^{14}C]palmitoyl-CoA as the substrate. The reaction mixture contained 1 mM of potassium cyanide. The reaction was stopped by the addition of perchloric acid.

Statistical analyses. Data were analyzed by one-way analysis of variance. When the corresponding F test for differences among test groups was significant, pairwise comparisons were made with the Dunnett's test ($p < 0.05$). The Bartlett's test for homogeneity of variance was also performed and if significant ($p < 0.005$), was followed by nonparametric procedures. Nonparametric procedures included the Kruskal-Wallis test for equal medians and the Mann-Whitney U test for pairwise comparisons ($p < 0.05$).

RESULTS

Body weights, food consumption, and survival. From test days 8 to 630, body weights were significantly decreased in the CP-C8, C8, and WY groups when compared to those of the *ad libitum* control group (Fig. 1). The decreases in body weights in the C8 and WY groups were primarily due to reduced food efficiency (Table 1). The overall mean daily intake values (test days 0–714) for the C8 and WY groups were 13.6 and 1.88 mg/kg/day, respectively (Table 1).

After 154 days on test, survival in the WY group decreased below the control group (Fig. 2). Gross examination revealed hemorrhages at several sites, which were attributed to a coagulopathy. The concentration of WY was decreased to 25 ppm on test day 301, and survival was subsequently stabilized. Due to this decreased survival, no WY-treated rats were sacrificed for biochemical or pathological evaluation at the 15-month time point. A discussion of the hematological changes has been previously published (Hurtt *et al.*, 1997). On test day 714,

TABLE 1
Effect of Chronic C8 and WY Exposure on In-Life Parameters

0–24 Months on test	*Ad libitum* diet	Pair-fed C8	300 ppm C8	50 ppm WY
Body weight gain (g)[a]	488.8 ± 126	407.7 ± 110	547.1 ± 158	427.7 ± 111
Food consumption (g)[b]	29.9	26.5	29.0	30.3
Food efficiency (g wt gain/g food consumed)[b]	0.023	0.022	0.026	0.020
Compound intake (mg/kg/day)	0	0	13.6	2.39

[a] Mean ± SD.
[b] Although these data were collected on an individual basis, data were reported on a group basis and therefore statistical analyses could not be performed.

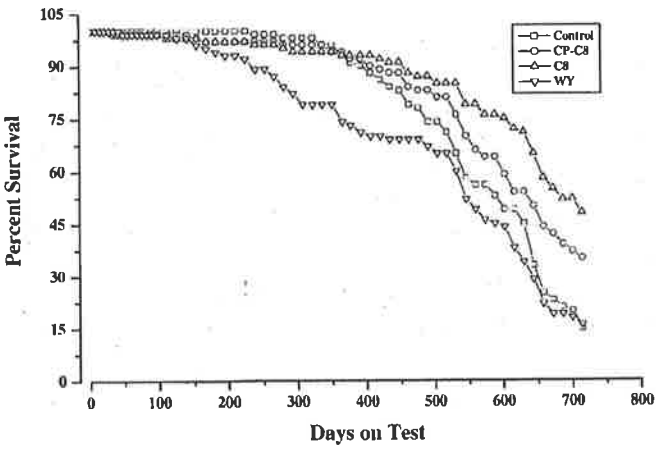

FIG. 2. Effect of C8 and WY on survival of male rats over the course of the 2-year feeding study. At test day 154, mean survival was decreased in male rats fed 50 ppm WY. The concentration of WY in the diet was decreased to 25 ppm on test day 301 and survival subsequently stabilized. On test day 714, survival was increased in the CP-C8 and C8 groups when compared to the *ad libitum* control.

survival was increased in the CP-C8 and C8 groups when compared to the control group (Fig. 2).

Liver. In the C8 and WY groups, relative liver weights (Fig. 3A) and hepatic β-oxidation activity (Fig. 3B) were increased at all of the sampling time points when compared to either the *ad libitum* or pair-fed control groups. The only exception was the C8 relative liver weight at 24 months, which was only significantly increased when compared to the pair-fed controls. In contrast, hepatic cell proliferation was only increased in the WY-treated rats (Fig. 3C). At 24 months, WY treatment resulted in increased incidence of hepatocellular adenomas (22% vs. 3% in the control group) and carcinomas (4% vs. 0% in the control group) (Table 2). Dietary administration of C8 produced a statistically significant increase in the incidence of hepatocellular adenomas (13% vs. 3% or 1% in the *ad libitum* or pair-fed control groups, respectively), but no carcinomas were observed in the C8 treated rats (Table 2).

Testis. Absolute testis weights were increased in the WY group at 21 and 24 months and in the C8 group at 24 months (Fig. 4A). Leydig cell β-oxidation activity (Fig. 4B) and Leydig cell proliferation (Fig. 4C) were not altered at any of the sampling times. Moreover, the rate of β-oxidation in Leydig cells, regardless of treatment, was approximately 20 times less than the rate of hepatic β-oxidation in the *ad libitum* or pair-fed control groups. At 24 months, dietary exposure to WY had significantly increased the incidence of Leydig cell hyperplasia (69% vs 14% in the control group) and adenomas (24% vs 0% in the control group) (Table 2). Dietary administration of C8 also produced increases in the incidence of Leydig cell hyperplasia (46% vs. 14% or 33% in the *ad libitum* or pair-fed control groups, respectively) and adenomas (11% vs. 0% or 3% in the *ad libitum* or pair-fed control group, respectively) (Table 2). There were no consistent changes observed for the weights of the epididymides, ASG unit with fluid, coagulating gland/seminal vesicle with fluid removed, or prostate throughout the study (data not shown).

Pancreas. Pancreatic acinar cell proliferation was increased in the C8 group at the 15-, 18-, and 21-month time points when compared the *ad libitum* or pair-fed control groups (Fig. 5). WY did not increase acinar cell proliferation at any time point. However, at 24 months, dietary exposure to WY had significantly increased the incidence of acinar cell hyperplasia (61% vs. 18% in the control group) and adenomas (37% vs. 0% in the control group) (Table 2). Dietary administration of WY did not produce any acinar cell carcinomas. Dietary administration of C8 also produced increases in the incidence of acinar cell hyperplasia (39% vs. 18% or 10% in the *ad libitum* or pair-fed control groups, respectively) and adenomas (9% vs. 0% or 1% in the *ad libitum* or pair-fed control groups, respectively). Additionally, a carcinoma was observed in one C8-treated rat.

Serum hormone measurements. Serum estradiol concentrations were significantly elevated in the C8-treated group at the 1-, 3-, 6-, 9-, and 12-month time points when compared to the *ad libitum* or pair-fed control groups (Fig. 6A). Serum estradiol concentrations were significantly elevated in the WY-treated group at the 3-, 6-, 9-, 18-, and 21-month time points when compared to the control group. After the 9-month time point, the dietary concentration of WY was reduced from 50 to 25 ppm, due to excessive mortality. At the 12-month sampling time point, serum estradiol concentrations in the WY group were similar to those of the control group, but were subsequently increased at 15, 18, and 21 months.

In contrast, C8 and WY did not alter serum testosterone concentrations in any consistent pattern (Fig. 6B). In the C8 group, serum LH was significantly elevated at the 6- and 18-month time points, and was numerically increased at the 9- and 12-month time points (Fig. 6C); serum FSH was significantly increased at the 6-month time point (Fig. 6D). In the WY group, serum LH was significantly elevated at the 6-, 12-, and 18-month time points, and was numerically increased at the 9- and 21-month time points (Fig. 6C). In the WY group, serum FSH was significantly increased at the 6- and 9-month time points, and was numerically increased at the 12-, 15-, 18-, and 21-month time points (Fig. 6D). C8 did not alter serum LH or FSH concentrations as consistently as WY, which is consistent with C8 being less potent than WY in producing Leydig cell tumors. Sustained elevation of serum LH has been reported to enhance Leydig cell tumorigenesis (Cook *et al.*, 1999). Although not always statistically significant, serum prolactin concentrations were numerically decreased in the WY group at the 1-, 3-, 6-, 9-, 12-, and 15-month time points and in the C8 group at the 1-, 3-, 6-, 9-, and 12-month time points (Fig. 6E). The prolactin data is difficult to interpret due to the high degree of variability. Subsequent work by the authors has shown that

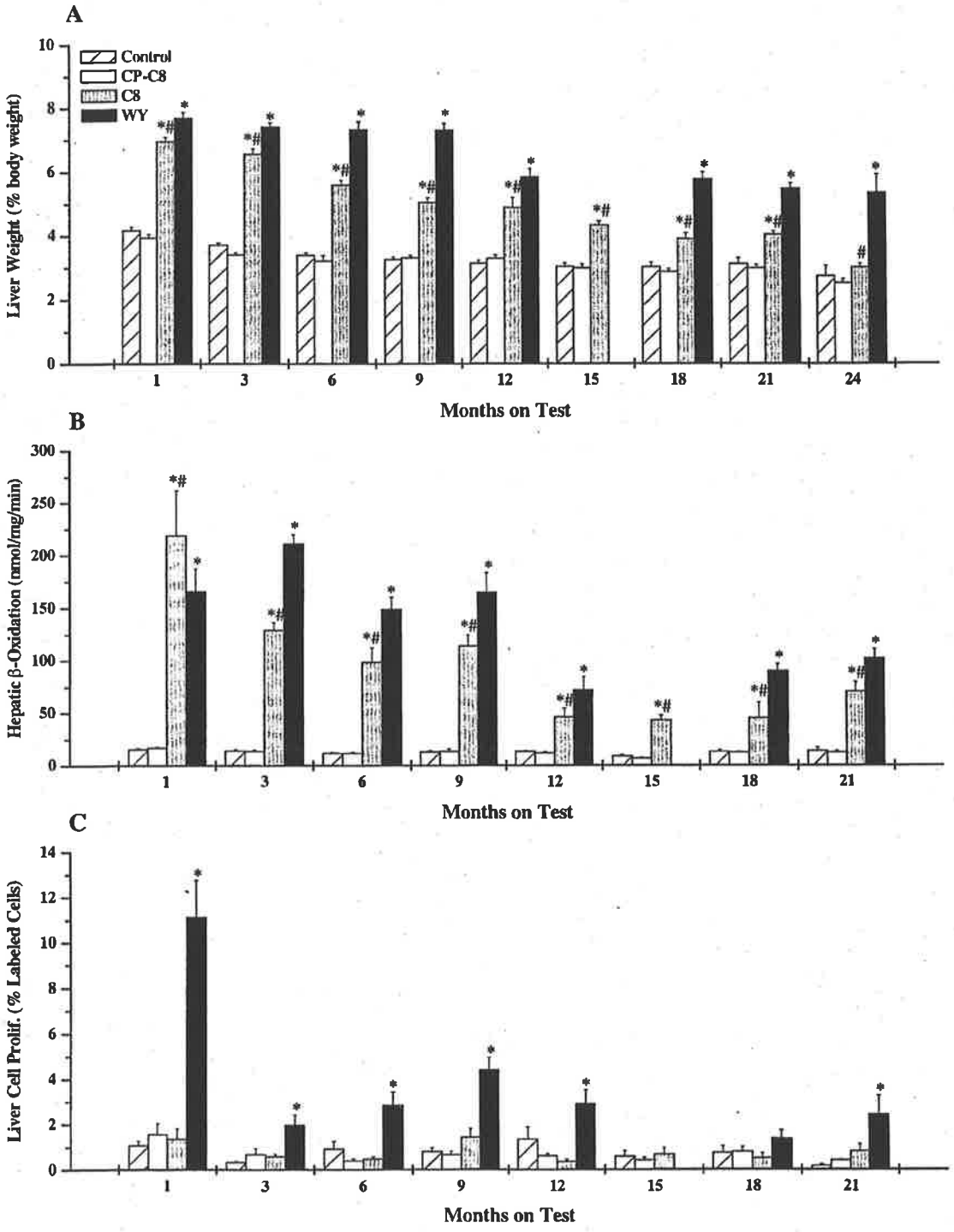

FIG. 3. Effect of C8 and WY on relative liver weights (A), hepatic β-oxidation activity (B), and hepatic cell proliferation indices (C) in male rats over the course of the 2-year feeding study. Relative liver weights and hepatic β-oxidation activity were increased at all sampling time points when compared to the control groups. In contrast, hepatic cell labeling indices were only increased in the WY-treated group. Data are reported as mean ± SD. Significantly different from the *ad libitum* control group ($^*p < 0.05$) or the pair-fed control group ($^\#p < 0.05$).

TABLE 2
Summary of Hyperplasia/Neoplasia Incidence in the Liver, Testes, and Pancreas from Rats Fed C8 or WY

Lesion	Control		CP-C8		C8 300 ppm		WY 25 ppm	
	Incidence	%	Incidence	%	Incidence	%	Incidence	%
Liver								
Adenoma	2/80	3	1/79	1	10/76	13[a]	15/67	22*
Carcinoma	0/80	0	2/79	3	0/76	0	3/67	4
Adenoma/carcinoma combined	2/80	3	3/79	4	10/76	13[a]	17/67	25*
Testes								
Leydig cell hyperplasia	11/80	14	26/78	33	35/76	46*	46/67	69*
Leydig cell adenoma	0/80	0	2/78	3	8/76	11[a]	16/67	24*
Pancreas								
Acinar cell hyperplasia	14/80	18	8/79	10	30/76	39[a]	41/67	61*
Acinar cell adenoma	0/80	0	1/79	1	7/76	9[a]	25/67	37*
Acinar cell carcinoma	0/80	0	0/79	0	1/76	1	0/67	0
Adenoma/carcinoma combined	0/80	0	1/79	1	8/76	11[a]	25/67	37*

Note. Values given for incidence of lesions are from all scheduled and unscheduled deaths; %, percent of control.
* Significantly different from the *ad libitum* control group, $p < 0.05$.
[a] Significantly different from the pair-fed control group, $p < 0.05$.

serum prolactin concentrations are affected by stress, and that the blood collection procedure utilized in the current study (i.e., tail vein blood collection) contributes to the variability (O'Connor *et al.*, 2000).

DISCUSSION

As hypothesized, both WY and C8 induced the tumor triad (i.e., hepatocellular, Leydig cell, and pancreatic acinar cell) in the current 2-year mechanistic study. WY increased the incidence of hepatocellular adenoma and carcinoma, Leydig cell hyperplasia and adenoma, and pancreatic acinar cell hyperplasia and adenoma, when compared to the control. A similar pattern was observed with C8, although C8 was clearly less potent than WY. For the liver effects, WY produced approximately a 2-fold greater incidence of combined (i.e., adenoma and carcinoma) tumors than C8, consistent with its ability to produce sustained increases in hepatic cell proliferation. These data are also consistent with the findings of Marsman and co-workers (1988) who demonstrated a similar relationship between di(2-ethlyhexyl)phthalate (DEHP) and WY. These studies illustrate how a sustained increase in cell proliferation can drive liver tumorigenesis. These data further demonstrate that peroxisome proliferators induce extrahepatic tumors (testis, pancreas), a relationship which has not been clearly demonstrated before. Several peroxisome proliferators produce extrahepatic tumors; however, these finding are only recently being addressed in review articles with this class of compound. For instance, clofibrate and HCFC-123, as well as C8 and WY, induce the tumor triad (liver, Leydig cell, and pancreatic acinar cell). Gemfibrozil, DEHP, and TCE induce liver and Leydig cell tumors (reviewed in Cook *et al.*, 1999), and Nafenopin induces liver and pancreatic acinar cell tumors (Reddy and Rao, 1997a,b).

Leydig Cell

Our early hypothesis for the mechanism of peroxisome proliferator-induced Leydig cell tumors was that this class of compounds increased peroxisomes in Leydig cells in a similar manner as in the liver (Cook *et al.*, 1992). This hypothesis was based on the similarity between hepatocytes and Leydig cells; both have abundant smooth endoplasmic reticulum; however, hepatocytes utilize this organelle for xenobiotic metabolism while Leydig cells utilize it for steroid biosynthesis. In 2 strains of rat, WY did not induce peroxisomes in Leydig cells based upon biochemical (peroxisomal β-oxidation activity) and electron microscopy (qualitative evaluation) criteria, at doses where abundant peroxisome induction was present in the liver (Biegel *et al.*, 1992; Hurtt *et al.*, 1992). In the current study, C8 and WY did not induce peroxisomes in Leydig cells, as measured by peroxisomal β-oxidation activity throughout the 2-year bioassay. These data demonstrate that peroxisome proliferators do not induce peroxisomes in Leydig cells, and hence, induce Leydig cell tumors via a different mechanism from that for liver tumors.

Early studies indicated that exposure to C8 and WY altered serum hormone concentrations. Surprisingly, in the current study, the only consistent alterations in serum hormone levels were an increase in estradiol concentrations and a mild decrease in prolactin concentrations; serum testosterone and LH concentrations were not significantly altered at the levels of C8 and WY that were tested. The Leydig cell tumors appear to be hormonally mediated where the sustained increase in estradiol, and possibly the decrease in prolactin concentrations, may play

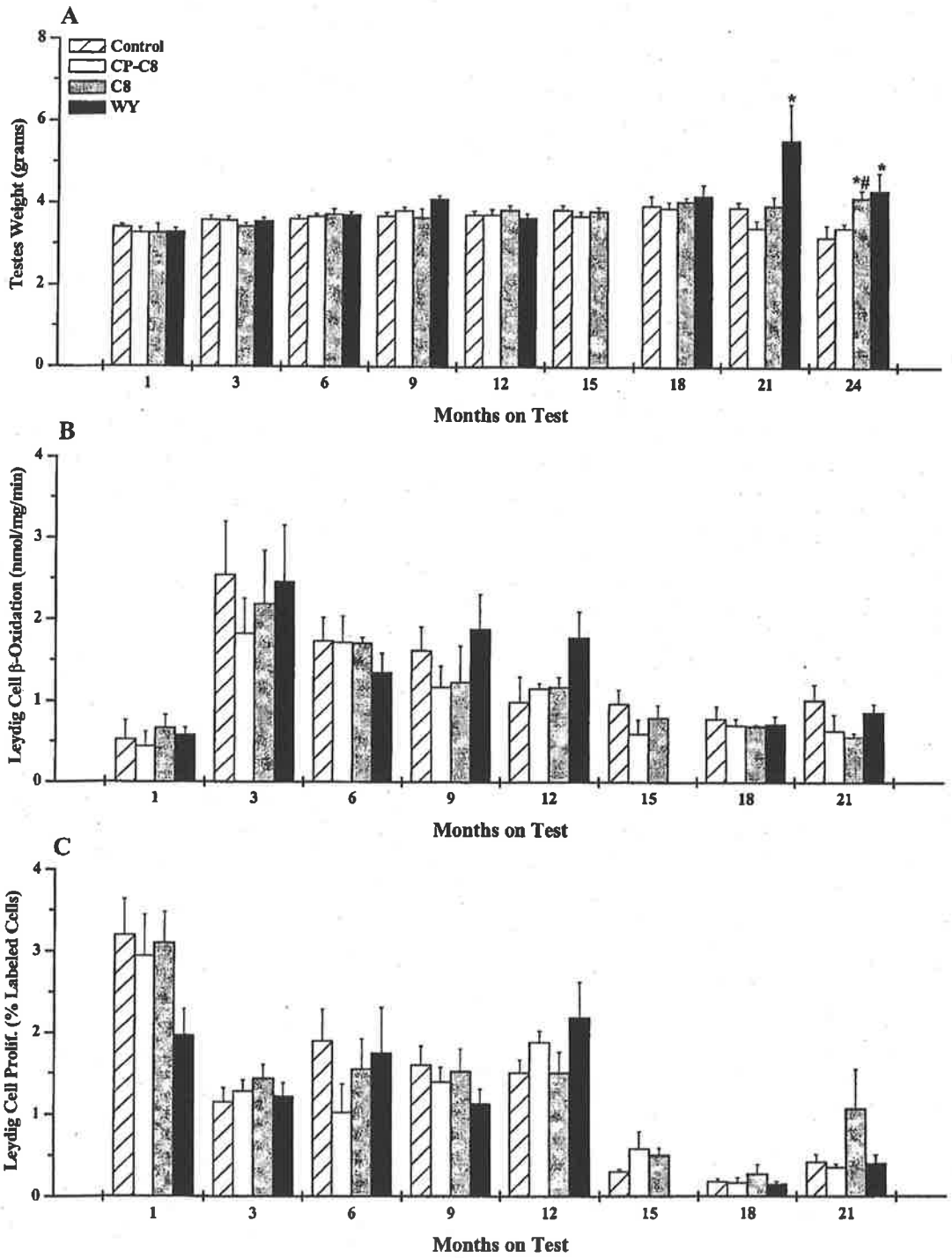

FIG. 4. Feeding study effect of C8 and WY on testes weights (A), Leydig cell β-oxidation activity (B), and Leydig cell proliferation indices (C) in male rats over the course of the 2-year study. Absolute testis weights were increased in the WY group at 21 and 24 months and in the C8 group at 24 months. This increase in testis weight was attributed to the increase in Leydig cell tumors. Leydig cell β-oxidation activity and cell proliferation were not altered at any time point. Data are reported as mean ± SD. Significantly different from the *ad libitum* control group ($*p < 0.05$) or the pair-fed control group ($^{\#}p < 0.05$).

a key role. Both C8 and WY produced biologically significant increases in serum estradiol concentrations after 1 month of dietary administration. While the increases in the current study were not always statistically significant, there were numerical increases in estradiol concentrations at every time point, which were considered biologically significant. The only exception

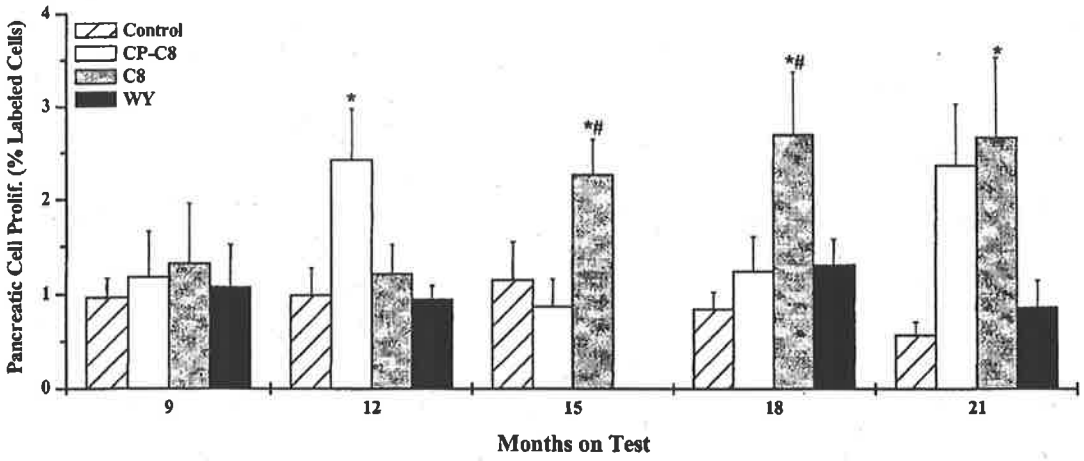

FIG. 5. Effect of C8 and WY on pancreatic acinar cell proliferation indices in male rats over the course of the 2-year feeding study. Pancreatic acinar cell proliferation was increased in the C8 group at 15, 18, and 21 months. Dietary administration of WY did not alter acinar cell proliferation. Data are reported as mean ± SD. Significantly different from the *ad libitum* control group ($*p < 0.05$) or the pair-fed control group ($^{\#}p < 0.05$).

was in the WY animals at the 12 month time point, where estradiol concentrations were not increased. However, this was attributed to the reduction in the dietary concentration of WY from 50 to 25 ppm that occurred on test day 301. The increase in serum estradiol in the WY group was reestablished at the 15-month time point and was maintained through the remainder of the study.

We have proposed a mechanism for the induction of Leydig cell tumors where estradiol modulates growth factor expression in the testis to produce Leydig cell hyperplasia and neoplasia (Biegel, *et al.*, 1995; Cook, *et al.*, 1992). Consistent with this hypothesis, WY produced approximately a 2-fold greater increase in the incidence of Leydig cell tumors than C8, and this correlated with the more sustained increase in estradiol that was observed in the WY-treated rats. In support of this hypothesis, it has been shown that administration of estradiol to mice produces Leydig cell tumors (Andervont *et al.*, 1960; Bonser, 1942; Hooker and Pfeiffer, 1942). In addition, it appears that human Leydig cell adenomas and the surrounding hyperplastic Leydig cells secrete large quantities of estradiol (Castle and Richardson, 1986; Due *et al.*, 1989). In male rats, serum estradiol concentrations are maintained by the conversion of testosterone to estradiol via aromatase, a cytochrome P450 containing monooxygenase (Coffey, 1988). It has been demonstrated that peroxisome proliferators increase serum estradiol levels via induction of aromatase (Biegel *et al.*, 1995, Liu *et al.*, 1996a,b). This hepatic aromatase induction increases serum estradiol concentrations (Biegel, *et al.*, 1995; Cook, *et al.*, 1992; Liu *et al.*, 1996a,b), which increases testis estradiol concentrations (Biegel, *et al.*, 1995). The increase in testicular estradiol concentrations (interstitial fluid) modulates growth factors, specifically TGFα, within the testis (Biegel, *et al.*, 1995).

Estradiol has been shown to stimulate the secretion of transforming growth factor (TGF-α) by mammary epithelial cells and over expression of TGFα has been suggested as one possible factor in producing sustained cell proliferation of mammary tumor cells and the subsequent development of neoplasia (Liu *et al.*, 1987). TGFα binds to the EGF receptor and stimulates cell proliferation (reviewed in Moses *et al.*, 1988). It is notable that TGFα stimulates thymidine incorporation into Leydig cell precursors and appears to be a Leydig cell stimulant (Khan *et al.*, 1992a). TGFα has been identified in Leydig cells (Teerds *et al.*, 1990). Hence, it is possible that the peroxisome proliferator-induced elevation of estradiol concentrations may be responsible for the development of Leydig cell adenomas. Studies with compounds that directly elevate serum estradiol concentrations (i.e., 17β-estradiol) are necessary to fully investigate this hypothesis.

Conflicting evidence exists for the role of estrogens in the development of Leydig cell tumors in rats. Estrogenic compounds do not induce Leydig cell tumors in rats when given at doses which produce testicular atrophy, which can confound detection of Leydig cell hyperplasia (Gibson, *et al.*, 1967; Marselos and Tomatis, 1992; Schardein, 1980; Schardein, *et al.*, 1970). These earlier studies were also limited by small sample size and reduced survival. Interestingly, GnRH agonists induce Leydig cell tumors at low doses, but do not induce Leydig cell tumors at higher doses where LH concentrations are suppressed and testicular atrophy occurs (Donaubauer *et al.*, 1987; Hunter *et al.*, 1982; Physician's Desk Reference, 1995a,b,c). Hence, these negative bioassays with estrogenic compounds may be due to suppression of LH, which to date is the primary demonstrated "driver" of Leydig cell tumors. Estradiol does appear to play a role in enhancement of Leydig cell tumorigenesis based on data from aging studies. In F344 rats, which have a high spontaneous incidence of Leydig cell tumors, there is an age-related increase in serum estradiol, which correlates with the development of Leydig cell hyperplasia and tumor formation (Turek and Desjardins, 1979). However, in

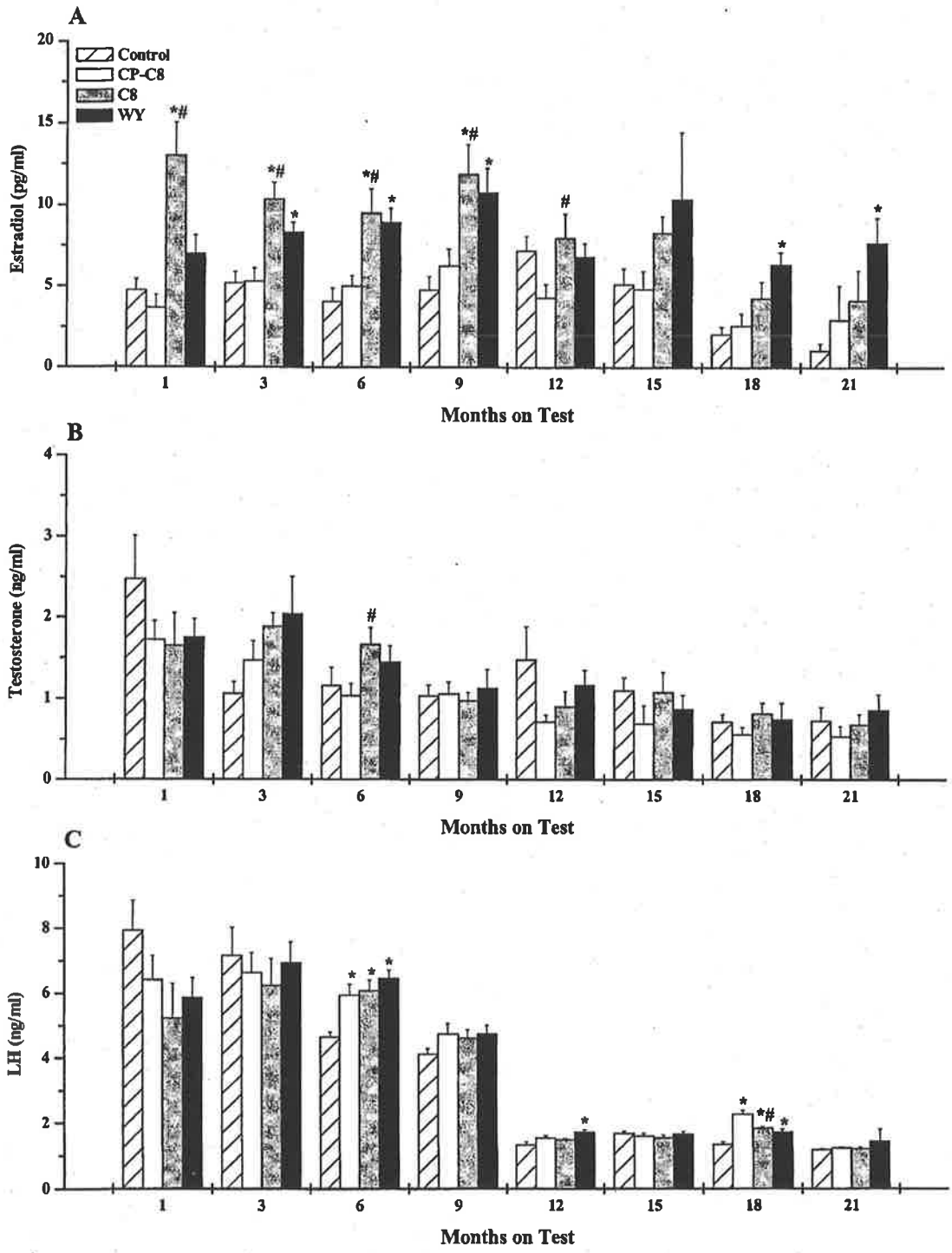

FIG. 6. Effect of C8 and WY on serum estradiol (A), testosterone (B), LH (C), FSH (D), and prolactin (E). Serum estradiol concentrations were elevated in the C8-treated group at 1, 3, 6, 9, and 12 months. Serum estradiol concentrations were elevated in the WY-treated groups at 3, 6, 9, 18, and 21 months. Serum testosterone was not altered by dietary exposure to C8 or WY. Although not always statistically significant, serum LH and FSH were numerically elevated in the WY group at 6, 9, 12, 18, and 21 months. Occasional elevations in LH and FSH were observed in C8-treated rats. Although not always statistically significant, serum prolactin concentrations were numerically decreased in the WY group at 1, 3, 6, 9, 12, and 15 months. A similar pattern was also observed in C8-treated rats. Data are reported as mean ± SD. Significantly different from the *ad libitum* control group ($*p < 0.05$) or the pair-fed control group ($^{\#}p < 0.05$).

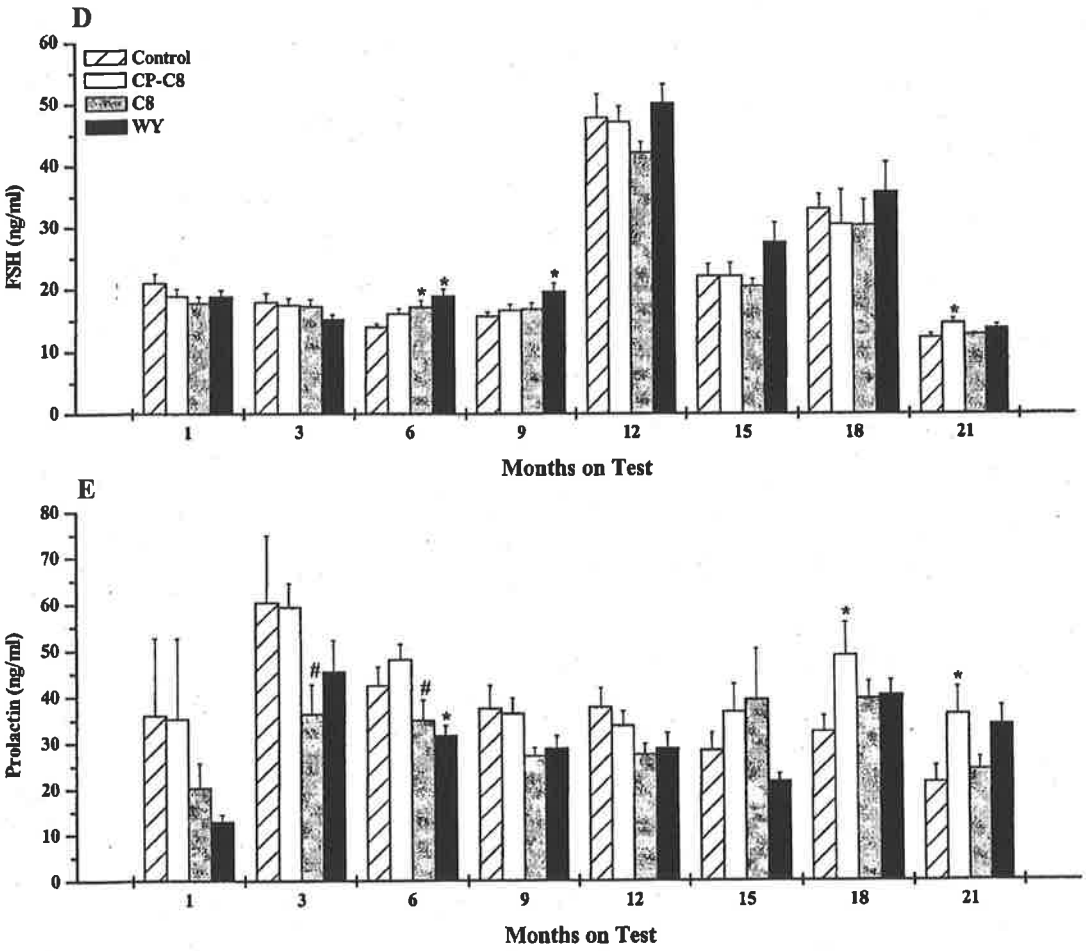

FIG. 6—*Continued*

the CD rat, which has a low spontaneous incidence of Leydig cell tumors, serum estradiol decreases with age (Cook *et al.*, 1994). In the current 2-year rat mechanistic bioassay, C8 and WY produced a sustained increase in serum estradiol concentrations that correlated with the potency of the 2 compounds to induce Leydig cell tumors. These studies suggest that estradiol may play a role in enhancement of Leydig cell tumors in the rat, and that peroxisome proliferators may induce Leydig cell tumors via a non-LH type mechanism. Whether estradiol plays a role in the induction of Leydig cell tumors by peroxisome proliferators can only be determined from an estradiol bioassay conducted at levels that do not induce testicular atrophy or reduce LH concentrations.

Pancreas

The development of pancreatic acinar cell tumors in the rat has been shown to be modified by several factors such as steroid concentrations (testosterone and estradiol), growth factors, cholecystokinin (CCK), and diet (fat) (Longnecker, 1983, 1987; Longnecker and Sumi, 1990). Castration, ovariectomy, and hormone replacement with estradiol and testosterone have been shown to influence the growth of carcinogen-induced preneoplastic foci in the azaserine-rat model of pancreatic carcinogenesis (Longnecker and Sumi, 1990). The incidence of spontaneous and induced neoplasms of the exocrine pancreas is higher in male than in female rats. Additionally, growth factors such as CCK have been shown to stimulate normal, adaptive, and neoplastic growth of pancreatic acinar cells in rats. CCK is found in the gut mucosa and is released into the bloodstream in response to the presence of food in the duodenum. CCK then binds to receptors on the pancreatic acinar cells and stimulates release of pancreatic secretions into the gut. The pancreatic secretions contain the monitor peptide, a protein that binds to the receptors in the duodenum to stimulate CCK release into the bloodstream. Chymotrypsin is also found in pancreatic juice and is cleaved into trypsin inside the gut. Trypsin digests proteins present in the gut. Once there is no food present in the gut, trypsin degrades the monitor

protein, which stops the further release of CCK. In the current 2-year study, WY produced approximately a 3.5-fold greater incidence of combined (i.e., adenoma and carcinoma) tumors than C8. The induction of pancreatic acinar cell tumors has also been reported for two other peroxisome-proliferating compounds, clofibrate and nafenopin (Physician's Desk Reference, 1996; Reddy and Rao 1997a,b). Hence, the induction of these tumors also appears to be associated with this class of compounds. It has also been shown that a series of aliphatic dicarboxylic acids, which produce hypolipidemic activity, increase fecal fat content. Although Izydore and Hall (1991) did not examine whether these aliphatic dicarboxylic acids are peroxisome proliferators, the "substrate overload hypothesis" would indicate that the dicarboxylic acids are responsible for the induction of peroxisomes. If this is true, then aliphatic dicarboxylic acids are likely to be peroxisome proliferators. Hence the ability of C8 and WY to induce pancreatic acinar cell tumors may be due to increasing the fat content in the gut, presumably by enhanced excretion of cholesterol/triglycerides in the liver. The increased fat content in the intestine would increase CCK release into the bloodstream. The sustained increase in serum CCK would enhance pancreatic acinar cell hyperplasia and the eventual formation of adenomas. Data suggest that peroxisome proliferators such as C8 and WY increase CCK concentrations; this may play a key role in the induction of pancreatic tumors. This hypothesis was further investigated by Obourn and co-workers (1997), who found that the WY-induced cholestasis produced increased plasma concentrations of CCK. They hypothesized that the pancreatic acinar cell tumors were induced via a mild, yet sustained increase in plasma CCK, secondary to hepatic cholestasis.

Summary

In conclusion, the peroxisome proliferators WY and C8 both produced the tumor triad of hepatocellular, Leydig cell, and pancreatic acinar cell tumors in the 2-year mechanistic bioassay in CD rats. This data, in conjunction with previously published data for other peroxisome-proliferating compounds (Cook *et al.*, 1992; Longnecker, 1983; Malley *et al.*, 1995; Tucker and Orten, 1995; Physician's Desk Reference, 1996; Reddy and Rao, 1997a) supports the hypothesis that induction of this tumor triad is a common occurrence among peroxisome-proliferating compounds. Regarding the induction of pancreatic acinar cell tumors, current data suggests that peroxisome-proliferating compounds such as WY and C8 induce pancreatic acinar cell tumors via increased CCK concentrations; however, the primary driver of the increased CCK has not been elucidated (Obourn *et al.*, 1997). The data from the current study suggest that the induction of the Leydig cell tumors by peroxisome proliferators is a result of a sustained increase in serum estradiol concentrations. Interestingly, GnRH agonists induce Leydig cell tumors at low doses, but do not induce Leydig cell tumors at higher doses where LH concentrations are suppressed and testicular atrophy occurs (Donaubauer *et al.*, 1987; Hunter *et al.*, 1982; Physician's Desk Reference, 1995a,b,c). Hence, these negative bioassays with estrogenic compounds may be due to suppression of LH, which to date is the primary demonstrated "driver" of Leydig cell tumors. In the current 2-year rat mechanistic bioassay, C8 and WY produced a sustained increase in serum estradiol concentrations that correlated with the potency of the 2 compounds to induce Leydig cell tumors. These studies suggest that estradiol may play a role in enhancement of Leydig cell tumors in the rat, and that peroxisome proliferators may induce Leydig cell tumors via a non-LH type mechanism. Whether estradiol plays a role in the induction of Leydig cell tumors by peroxisome proliferators can only be determined from an estradiol bioassay conducted at levels that do not induce testicular atrophy or reduce LH concentrations.

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DOCUMENT 54

CONSENT ORDER ISSUED PURSUANT TO ARTICLES 5 and 12, CHAPTER 22 AND ARTICLE 1, CHAPTER 16 OF THE WEST VIRGINIA CODE.

TO: E. I. DU PONT DE NEMOURS AND COMPANY DATE: November 14, 2001

West Virginia Department of Environmental Protection **Order No. GWR-2001-019**
West Virginia Department of Health and Human Resources

This **CONSENT ORDER** is issued by the Director of the Division of Water Resources and Director of the Division of Air Quality, West Virginia Department of Environmental Protection, and the Commissioner of the Bureau for Public Health, West Virginia Department of Health and Human Resources, pursuant to the authority set forth in more detail below.

I. INTRODUCTION OF PARTIES.

This Consent Order is entered into by and between the West Virginia Department of Environmental Protection [WVDEP], the West Virginia Department of Health and Human Resources – Bureau for Public Health [WVDHHR-BPH], and E. I. du Pont de Nemours and Company [DuPont][collectively referred to as the "Parties"].

II. PURPOSE OF CONSENT ORDER.

This Consent Order sets forth a series of tasks to be performed by the Parties in order to determine whether there has been any impact on human health and the environment as a result of releases of ammonium perfluorooctanoate [C8], CAS Number 3825-26-1, to the environment from DuPont operations. C8 is a material used by DuPont in its fluoroproducts manufacturing process at its Washington Works facility located at Washington, Wood County, West Virginia. C8 is not identified as a hazardous substance, hazardous waste or otherwise specifically regulated under West Virginia or federal statute or regulation.

This Consent Order has been negotiated in good faith and the actions undertaken by DuPont pursuant to this Consent Order do not constitute an admission of any liability on its part. DuPont retains the right to controvert in any other proceedings, other than proceedings to implement or enforce this Consent Order, the validity of the findings of fact and conclusions of law set forth herein. DuPont agrees to comply with and be bound by the terms of this Consent Order and further agrees in any proceeding to implement or enforce this Consent Order that it

will not contest the validity of this Consent Order or the jurisdiction of WVDEP and WVDHHR-BPH to issue it.

III. DEFINITIONS.

Whenever the terms identified below are used in the Consent Order or in any exhibit or attachment hereto, the following definitions shall apply:

1. "The Agencies" shall mean the Department of Health and Human Resources, Bureau for Public Health and the Department of Environmental Protection, including the Divisions of Air Quality and Water Resources.

2. "C8" shall mean the chemical compound ammonium perfluorooctanoate.

3. "Detection Limit" means the lowest analytical level that can be reliably achieved within specified limits of precision and accuracy under routine laboratory conditions for a specified matrix. It is based on quantitation, precision and accuracy under normal operation of a laboratory and the practical need in a compliance-monitoring program to have a sufficient number of laboratories available to conduct the analyses.

4. "Effective Date" shall mean the date set forth in Section XVII of this Consent Order.

5. "EPA" shall mean the United States Environmental Protection Agency.

6. "Force Majeure" shall mean conditions or circumstances beyond the reasonable control of DuPont which could not have been overcome by due diligence and shall include, without limitation, acts of God, action or inaction of governmental agencies, or administrative or judicial tribunals or other third parties, or strikes or labor disputes (provided, however, DuPont shall not be required to concede to any labor demands), which prevent or delay DuPont from complying with the work plan.

7. "Groundwater Monitoring Well" shall mean any cased excavation or opening into the ground made by digging, boring, drilling, driving, jetting, or other methods for the purpose of determining the physical, chemical, biological, or radiological properties of groundwater. The term "monitoring well" includes piezometers and observation wells, which are installed for purposes other than those listed above, but does not include wells whose primary purpose is to provide a supply of potable water.

8. "Groundwater Well" or "Well" shall mean any drilled or excavated groundwater collection system that supplies water for public, private, industrial, or agricultural use and shall include drinking water wells. As used in this Consent Order, this term applies only to wells

located in West Virginia.

9. "Reimbursable Costs" shall mean costs attributable (on an hourly basis) to the work of Dee Ann Staats, Ph.D. in the negotiation and implementation of this Consent Order, the costs attributable to any other participants on the C8 Assessment of Toxicity Team, as described in Attachment C to this Consent Order, who are serving in that position as contractors to WVDEP, costs incurred by WVDEP in connection with the public meetings described in Attachment C, and costs attributable to any contractor retained at the direction of the Groundwater Investigation Steering Team (GIST).

10. "Washington Works" shall mean the manufacturing facility owned by DuPont and located in Washington, Wood County, West Virginia, as depicted on Exhibit 1 to this Consent Order.

11. "The Facilities" shall mean the Washington Works and the Local Landfill, depicted on Exhibit 1, the Letart Landfill, depicted on Exhibit 2, and the Dry Run Landfill, depicted on Exhibit 3.

12. "Reference Dose" or "RfD" shall mean an estimate (with uncertainty spanning perhaps an order of magnitude or greater) of a daily exposure level for the human population, including sensitive subpopulations, that is likely to be without an appreciable risk of deleterious effects during a lifetime. Chronic RfDs are specifically developed to be protective for long-term exposure to a compound.

13. "Screening Level" shall mean the concentration in a specific media such as air, water, or soil, that is likely to be without an appreciable risk of deleterious effects during a lifetime in the human population.

IV. WAIVER OF RIGHTS.

DuPont waives any and all rights it may have to appeal or challenge the validity or requirements of this Consent Order, and shall not challenge the jurisdiction of the Agencies to issue this Consent Order.

This Consent Order applies to and is binding upon the Parties, and their successors and assigns.

V. FINDINGS OF FACT.

1. C8 is a chemical substance which has no established state or federal effluent or emission standards.

2. C8 is a perfluorinated surfactant manufactured by the 3M Company and others.

Since the early 1950's C8 has been used by DuPont in its fluoropolymer-related manufacturing processes at its Washington Works facility, located in Wood County, West Virginia.

3. Residues containing C8 from fluoropolymer manufacturing processes at Washington Works are or have been released to the air, discharged to the Ohio River, disposed of at the Facilities, and otherwise shipped off-site for destruction and/or disposal. DuPont also captures for recycle a significant portion of used C8.

4. No permits issued to DuPont authorizing releases of pollutants to the environment contain specific limitations on the amount of C8 that may be released to the environment. However, C8 releases are addressed more generally in WVDEP Division of Air Quality permits as particulate matter, PM_{10} (particulate matter with an aerodynamic diameter less than or equal to 10 microns), or as a volatile organic compound.

5. Since as early as 1990, DuPont has performed regular, voluntary water sampling to detect the presence and level of C8 in and around certain of its Facilities in West Virginia and has reported the results of this sampling to various government agencies. Currently, DuPont also samples and reports C8 concentrations in water as required by permits issued by WVDEP and EPA.

6. As a result of DuPont's sampling, C8 has been detected in varying concentrations in and around certain of its Facilities in West Virginia, including private drinking water wells and public water supplies.

7. Analyses of water samples have reported levels of C8 in the Lubeck Public Service District ("LPSD") drinking water supply.

8. DuPont, by and through its use of C8 in the fluoropolymer manufacturing process, is the likely source of C8 presence in and around certain of its Facilities in West Virginia.

9. Along with environmental sampling for C8, DuPont has performed and participated in multiple studies examining the potential effects of C8 exposure on human health and the environment.

10. Studies performed by DuPont and 3M have determined that C8 in sufficient doses, i.e., considering both amount and duration of exposure, is toxic to animals through ingestion, inhalation and dermal contact. Studies have also found that C8 is persistent in humans and the environment.

11. Although DuPont has collected a large amount of data on the presence of C8 in the environment, the Agencies believe that additional information will assist them in delineating the extent and concentrations of C8 in the environment at or near the Facilities. Available data collected by DuPont indicates that C8 is present in the surface and groundwater at the Letart and

Dry Run Landfills and at or near the Washington Works facility.

12. WVDEP and WVDHHR-BPH have determined that it is desirable to ascertain the source of drinking water for persons potentially exposed to C8 in groundwater or surface waters in the area of the Facilities.

13. EPA, WVDEP, and WVDHHR-BPH, in consultation and cooperation with one another, have requested, and DuPont has submitted, information and documents relating to the detection and presence of C8 in and around the Facilities and documents with respect to the human health studies being performed related to C8 exposure.

14. Based upon information submitted by DuPont and reviewed to date by EPA, WVDEP, and WVDHHR-BPH, the Agencies believe that additional data would assist in their evaluation of whether the ground and surface waters now containing C8 have a complete exposure pathway to humans and whether persons in and around the Facilities are at risk of adverse health effects from C8 exposure.

15. There have been no independent governmental or non-industrial studies performed on the human health effects of C8 exposure for the purpose of establishing an exposure standard for C8 applicable to the general public.

16. The Agencies have concluded that full site and health assessments are necessary to ascertain the extent and level of C8 concentrations in the environment and to assist them in determining whether C8 presents any possible danger to the public. DuPont has agreed to participate and assist in this effort.

17. The fluoropolymers industry has committed to EPA to reduce total actual C8 emissions for either the year 1999 or the year 2000 by 50 percent within three to five years of each company's commitment date. DuPont committed to this goal in 2000.

18. DuPont installed, in March 2001, a filter and carbon treatment system at its Washington Works facility that is demonstrating removal efficiency of 90-95% of the C8 in its major C8-containing wastewater stream.

VI. AUTHORITY TO ISSUE CONSENT ORDER.

1. The WVDEP is the state agency vested with the authority to protect the environment in West Virginia.

2. Article 12, Chapter 22 of the West Virginia Code, the Groundwater Protection Act, grants to the WVDEP the authority to protect the State's groundwater from any contaminant

and, where contaminated groundwater is found, to institute a civil action or issue an order requiring that groundwater be remediated.

3. Article 5, Chapter 22 of the West Virginia Code, the Air Pollution Control Act, grants to the WVDEP the authority to protect the State's air from pollutants and to institute a civil action or issue orders to enforce the statute.

4. The WVDHHR-BPH is the state agency vested with the authority to regulate and protect drinking water supplies in West Virginia.

5. Article 1, Chapter 16 of the West Virginia Code, grants to the WVDHHR-BPH the authority to protect the public drinking water supply of the state and to perform all investigation necessary to assure its purity and safety, and further grants to the WVDHHR-BPH the authority to institute actions and issue orders to restore the purity of said water supply.

VII. REQUIREMENTS OF CONSENT ORDER.

The Agencies have concluded that it is of great importance to have sufficient data upon which to determine the scope and potential risk of the presence of C8 in the environment in and around the Facilities. Therefore, the Agencies require the following:

A. Establishment of Groundwater Investigation Steering Team.

1. A "Groundwater Investigation Steering Team" (GIST) shall be established with members of the team consisting of WVDEP, WVDHHR-BPH, EPA Region III, and DuPont. The WVDEP representative will be the team leader. The objectives and specific tasks of the team are set forth in full in Attachment A of this Consent Order. However, the primary purpose of the GIST will be to oversee an expeditious, phased approach to fulfilling the majority of the requirements set forth in Sections A through C. The work performed with oversight from the GIST shall be funded by DuPont in accordance with Section VIII of this Consent Order.

2. Upon conclusion of key milestones in the tasks set forth in Attachment A, the GIST shall issue interim or final reports setting forth findings of fact and conclusions regarding background data, groundwater monitoring, and plume identification as described in Attachment A. Any groundwater monitoring plan developed pursuant to Attachment A shall survive the termination of this Consent Order and shall be incorporated as a minor permit modification for the Facilities. DuPont reserves the right to request modification of the plans upon renewal of the Facilities' permits.

B. National Pollutant Discharge Elimination System Requirements.

1. Except as occasioned by no-flow conditions, DuPont shall perform monthly sampling for C8 at the Local Landfill at certain outfalls identified in West Virginia/National Pollutant Discharge Elimination System ("WV NPDES") Permit No. 0076538 as Outfalls 101, 004 and 005.

2. Except as occasioned by no-flow conditions, DuPont shall perform monthly sampling for C8 at the Washington Works facility at certain outfalls identified in WV NPDES Permit No. WV0001279 as Outfalls 001, 002, 003, 005, 007, and 105.

3. Except as occasioned by no-flow conditions, DuPont shall perform monthly sampling for C8 at Dry Run Landfill at all outfalls identified in its WV NPDES Permit No. WV0076244.

4. Except as occasioned by no-flow conditions, DuPont shall perform monthly sampling for C8 at Letart Landfill at all outfalls identified in its WV NPDES Permit No. WV0076066.

5. With respect to the requirements of paragraphs VII.B.1 through VII.B.4, all sampling shall be performed pursuant to established EPA guidelines, where applicable, and results shall be delivered to the WVDEP within thirty days of receiving such results. DuPont shall record and report all attempts to sample under no-flow conditions.

6. Within 90 days of the Effective Date of this Consent Order, DuPont agrees to obtain a sample from each surface or alluvial water intake for public water supplies along the Ohio River in the area extending ten river miles downstream of the Washington Works facility and one river mile upstream of the Washington Works facility. If concentrations of C8 above the Detection Limit are found in any sampled public water supply within the upstream or downstream segments initially sampled, the segments within which intakes are to be sampled shall be extended to twenty river miles downstream or two river miles upstream, as appropriate. If concentrations above the Detection Limit are found in any segment so extended, additional sampling will be performed on water intakes within thirty river miles downstream or three river miles upstream, as appropriate.

7. The additional monitoring requirements contained in this subsection shall be incorporated into the Facilities' West Virginia/National Pollutant Discharge Elimination System permits by minor modification. DuPont reserves the right to request a modification of these requirements upon renewal of the permits.

C. Toxicological and Human Health Assessment.

1. DuPont agrees to fund the various tasks set forth below as a part of this Consent Order by establishing an escrow account at a bank agreed to by the Parties, or by some other

means agreed to by the Parties. Disbursements from said escrow shall be authorized by the C8 Toxicity Team Leader and DuPont representative jointly as described below.

2. A C8 Assessment of Toxicity Team ("CAT Team") shall be established with members of the team consisting of representatives of:

WVDEP
WVDHHR-BPH
EPA Region III
NICS
ATSDR
DuPont

3. The WVDEP representative shall be the Team Leader.

4. The individual team members, the tasks of the team, and the team objectives are set forth in full in Attachment C of this Consent Order.

5. Upon conclusion of all the tasks set forth in Attachment C, the CAT Team shall issue a final report setting forth findings of fact and conclusions as to what extent there may be health risks associated with C8 at the Facilities.

D. Emission Modeling Assessment.

1. The following information shall be submitted to the Division of Air Quality ("DAQ") within 30 days of the Effective Date except where a different deadline is provided in this subsection:

a. A complete and accurate list of building dimension parameters for all structures located within the Washington Works facility that have a significant impact on the dispersion of C8 emissions. Significant impact for each structure on the site shall be determined based on the "area of building wake effects" as defined in the EPA User's Guide to the Building Profile Input Program (EPA-454/R-93-038 Revised Feb. 8, 1995).

b. A complete and accurate list of DuPont's current permitted allowable emission rates and confirmed actual C8 emission rates in pounds per year for the year 2000 for all sources located within the Washington Works facility. Each emission point shall be listed according to its stack I.D. and corresponding permit number. For each stack identified above as emitting C8 DuPont shall list all relevant stack parameters to be used in air dispersion modeling.

c. For each emission point (stack) emitting C8, the following information shall be supplied:

i. Phase of C8 (solid, vapor or aqueous solution) at stack conditions.

ii. The particle characterization to be used for modeling including the particle size distribution (microns), the mass fraction of C8 in each particle size category, and the particle density (g/cm^3).

iii. For particulate emissions, scavenging coefficients (hr/s-mm) for both liquid and frozen precipitation to be used for wet deposition modeling based upon the particle size distribution and the EPA's Industrial Source Complex, Version 3 Model Guidance (EPA-454/B-95-003b Sept. 1995) ("ISC Guidance"). DuPont may submit, within 30 days of the Effective Date, information to support the use of the normalized scavenging coefficient in the ISC Guidance (Figure 11 of ISC Guidance) for C8's scavenging coefficients. DAQ shall approve or disapprove with justification in writing, DuPont's submission. Should DAQ disapprove, DuPont shall have the right, within seven days, to request a meeting with DAQ and USEPA to address the deficiencies set forth in DAQ's letter and to request reconsideration of DAQ's decision. Following a meeting of the parties, DAQ shall issue a decision letter regarding C8's scavenging coefficients within seven days of the meeting. DAQ reserves the right to require measurement of C8's scavenging coefficients in its decision and DuPont reserves the right to assert a claim of confidentiality in the event such a measurement is made.

iv. For gaseous emissions, scavenging coefficients (hr/s-mm) for both liquid and frozen precipitation to be used for wet deposition modeling will be provided as a function of droplet size using formulae in the open literature based on the physical properties of C8 and consistent with Section 1.4 of the ISC Guidance. DuPont may submit, within 30 days of the Effective Date, information to support the proposed scavenging coefficient for gaseous emissions including information on the percentage of C8 emissions that would be in gaseous form. DAQ shall approve or disapprove with justification in writing, DuPont's submission. Should DAQ disapprove, DuPont shall have the right, within seven days, to request a meeting with DAQ and USEPA to address the deficiencies set forth in DAQ's letter and to request reconsideration of DAQ's decision. Following a meeting of the parties, DAQ shall issue a decision letter regarding C8's scavenging coefficients within seven days of the meeting. DAQ reserves the right to require measurement of C8's scavenging coefficients in its decision and DuPont reserves the right to assert a claim of confidentiality in the event such a measurement is made.

d. To the extent that the phases exist, a solid, liquid and vapor phase (T-P) diagram for C8 with respect to pressure and temperature. The temperature and pressure ranges shall be representative of exhaust gas conditions before and after control equipment. Estimates of C8's critical properties shall be provided along with measured ranges of phase transition temperatures.

e. In lieu of a binary phase (T-x-y) diagram representing the vapor-liquid equilibrium between water and C8, the solubility and Krafft Point of C8 in aqueous solutions, measured pK value for C8 dissociation in aqueous solutions, and measurements of C8 concentrations or related acids observed when tested in a head space GC at various concentrations, temperatures, and pHs representative of the ranges observed during actual operating conditions. Furthermore a discussion regarding the volatility of C8 in aqueous solutions as a function of pH will be provided. The information in this paragraph shall be submitted to the DAQ within 60 days of the Effective Date.

f. Henry's law coefficient for C8 and a discussion of its dependence on pH. The coefficient shall be defined at various temperatures covering the range observed during actual operations.

g. Any carbon adsorption data in the form of isotherms for C8 adsorption.

DAQ will provide DuPont an opportunity to comment on modeling methodology and assumptions prior to finalizing the modeling results.

2. Any expenses incurred as a result of accurately supplying the information requested above shall be covered by DuPont.

3. Upon submission of the information required by this Subsection VII.D, DAQ reserves the right to disapprove any data if the analytical methodology or quality control procedures are deemed inappropriate.

VIII. REIMBURSEMENT OF COSTS.

1. DuPont agrees to establish an escrow account to fund Reimbursable Costs under this Consent Order. Expenditures from this account shall be made upon joint approval by a duly designated representative of the WVDEP and of DuPont ("designated representatives"). Written notice of such designation shall be sent to the persons identified pursuant to Section XVI of this Consent Order. Prior to the execution of this Consent Order, WVDEP has provided DuPont with an estimate of Reimbursable Costs that WVDEP expects to incur under this Consent Order.

2. Within 10 business days of the Effective Date, DuPont shall deposit in the escrow account funds in the amount of fifty thousand dollars ($50,000). Each expenditure from the escrow account must be supported by an itemized accounting, including invoices and receipts. Said escrow account shall be replenished with additional funds whenever the balance is less than ten thousand dollars ($10,000), or as agreed to by the designated representatives. Any unexpended amount remaining in the escrow account at the conclusion of the work to be performed under this Consent Order shall be returned to DuPont.

3. DuPont's obligation to pay Reimbursable Costs under this Consent Order shall

not exceed two hundred and fifty thousand dollars ($250,000). Except as to Reimbursable Costs which are addressed separately in this section, all other costs incurred by DuPont in carrying out its obligations under Consent Order shall be the sole responsibility and obligation of DuPont.

IX. QUALITY ASSURANCE/QUALITY CONTROL.

All sampling and analyses performed pursuant to this Consent Order shall conform to EPA guidance regarding quality assurance/quality control, data validation, and chain of custody procedures. The laboratory performing the analyses shall be approved by the Parties prior to sampling.

X. C8 REDUCTION PROGRAM.

1. Notwithstanding current permitted emission levels, DuPont agrees to limit overall C8 emissions to the air to no more than actual calendar year 2000 levels on a calendar year basis and shall further provide to the WVDEP monthly emissions reports regarding C8. The reporting requirement contained herein shall be modified to quarterly reports upon the issuance of a Screening Level derived following the procedures set out in Attachment C.

2. DuPont agrees to reduce emissions to the air and discharges to the water of C8 collectively by 50% from actual 1999 levels by December 31, 2003.

3. DuPont shall operate and maintain the filter and carbon bed treatment system at its Washington Works facility with the goal of achieving 90-95% C8 removal efficiency in its major C8-containing wastewater stream.

4. DuPont shall conduct the following construction projects and abide by the specified dates:

a. DuPont shall install an improved scrubber filter to replace recovery device T6IZC on permit R13-815D. Construction shall begin no later than February 28, 2002. Initial operation shall begin no later than the date of start up after the April shutdown, or June 28, 2002, whichever is earlier.

b. DuPont shall modify the stack for emission point T6IZCE so that the emission point elevation is 170 feet above grade. The stack diameter, velocity, and flow rate shall be sized to provide effective dispersion of particulate emissions according to 45 Code of State Rules, Series 20 (Good Engineering Practice as Applicable to Stack Heights). Construction shall begin no later than February 28, 2002. Initial operation shall begin no later than the date of start up after the April shutdown, or June 28, 2002, whichever is earlier. At times when device T6IZC is not operating, permitted emissions from scrubber T6IFC shall be emitted to emission point

T6IZCE.

5. DuPont shall conduct a scrubber optimization and recovery improvement program that shall consist of a study of scrubber operation for device C2DWC2 on permit R13-614A. The study shall be complete by the end of March 2002. Provided the results are encouraging, the company shall implement identified improvements for this device and similar improvements for units C2DTC2 on permit R13-614A, C2EHC2 on permit R13-1953, and C1FSC2 on proposed permit for R13-2365A. Implementation of the improvements for the latter devices will be complete no later than the end of November 2002.

XI. COMPLIANCE WITH SCREENING LEVELS.

1. The following requirements shall apply only if the procedures set out in Attachment C have been followed:

a. No later than 60 days after receipt of notification from the Agencies that data or information developed pursuant to this Consent Order or other information that is recent and valid demonstrates that DuPont's operations have resulted in C8 exposures above the Screening Levels derived following the procedures set out in Attachment C, DuPont shall submit a plan for review and approval by the Agencies that is designed to reduce such exposures to levels below the Screening Levels within a reasonable time (the "Remedial Plan" or "the Plan").

b. Within 30 days of receipt of the Remedial Plan submitted by DuPont, the WVDEP shall, upon consultation with the WVDHHR-BPH and based upon accuracy, quality, and completeness, either approve or disapprove the Plan. If the WVDEP disapproves the Remedial Plan, the WVDEP shall notify DuPont in writing that the Remedial Plan has been disapproved and shall specify the reasons for such disapproval. DuPont shall resubmit the Remedial Plan as revised to address the deficiencies identified in the notice. DuPont's failure to submit an approvable Remedial Plan shall be deemed a violation of this Consent Order.

2. In the event EPA or the WVDEP develops and finalizes a reference dose/screening level for C8 in accordance with applicable statutory and regulatory requirements ("the Regulatory EPA Standard") that would be applicable to Dupont's activities or the Facilities independent of this Consent Order, DuPont's obligations under this Section shall be determined with reference to the Regulatory EPA Standard. DuPont reserves all rights it may have to comment upon, object to, or appeal the Regulatory EPA Standard in proceedings separate and apart from this Consent Order.

XII. COMPLETION OF CONSENT ORDER.

1. Except as to DuPont's obligations under Section XI, this Consent Order and DuPont's obligations hereunder shall terminate upon issuance of a completion letter(s) from the Secretary of the WVDEP or his designee and from the Commissioner of the WVDHHR-BPH to

DuPont. In a timely manner following receipt of a written request from DuPont the respective Agencies shall issue the completion letter(s) to DuPont or shall issue a letter to DuPont detailing the obligations and work that have not been completed in accordance with this Consent Order. The Parties agree that the Agencies' obligation to issue this letter shall be deemed a non-discretionary duty.

2. DuPont's obligation to achieve and maintain compliance with the Screening Levels as provided in Section XI of this Consent Order shall survive the termination of this Consent Order. Such obligation shall terminate only as provided in Section XI or upon agreement of the Parties.

XIII. ADDITIONAL ACTIONS.

The Agencies, individually or collectively, pursuant to their statutory duty and authority, may determine that additional action, beyond the tasks set forth in this Consent Order, is necessary to protect human health and/or the environment. Nothing in this Consent Order shall be construed as restraining or preventing the Agencies from taking such actions. Nothing in this Consent Order constitutes a satisfaction of or release from any claim or cause of action against DuPont for any liability it may have pursuant to the federal Clean Water Act, the federal Clean Air Act, the federal Safe Drinking Water Act, the West Virginia Groundwater Protection Act, the West Virginia Air Pollution Control Act, other statutes applicable to this matter, or West Virginia common law. Nothing in this Consent Order in any way constitutes a modification or waiver of statutory requirements of DuPont and nothing in this Consent Order shall obligate DuPont to undertake any actions not specified herein.

XIV. ENFORCEMENT.

Enforcement of this Consent Order may be had by the filing of a civil action by any of the Agencies in the Circuit Court of Wood County, West Virginia. Violation of the terms and conditions of this Consent Order by DuPont is a violation of the West Virginia Code and may result in enforcement action being taken, including a request for civil penalties as set forth by law. DuPont shall not be liable for violations of this Consent Order due to any "Force Majeure" condition.

XV. CONTENTS OF CONSENT ORDER/MODIFICATION.

The entirety of this Consent Order consists of the terms and conditions set forth herein and in any attachments or exhibits referenced herein. Modification of the terms and conditions of this Consent Order including any modification of timeframes or deadlines established in this Consent Order shall be made only by agreement of the Parties in writing, except that modifications to any

requirement set out in the attachments to this Consent Order may be made upon consensus of the members of the GIST or the CAT Team, as appropriate.

XVI. ADDRESSES FOR ALL CORRESPONDENCE

All documents, including reports, approvals, notifications, disapprovals, and other correspondence, to be submitted under this Consent Order shall be sent by certified mail, return receipt requested, hand delivery, overnight mail or by courier service to the following addresses or to such addresses DuPont or WVDEP may designate in writing.

Documents to be submitted to WVDEP should be sent to:

WV Department of Environmental Protection
1356 Hansford Street
Charleston, West Virginia 25301

Attention: Armando Benincasa, Esq.
Attention: Dee Ann Staats, Ph.D.
Phone No.: (304) 558-2508

Documents to be submitted to WVDHHR-BPH should be sent to:

WV Department of Health and Human Resources
Bureau for Public Health
815 Quarrier Street, Suite 418
Charleston, West Virginia 25301

Attention: William Toomey, Manager of Source Water Assessment Program
Phone No.: (304) 558-2981

Documents to be submitted to DuPont should be sent to:

E. I. du Pont de Nemours and Company
Washington Works
P.O. Box 1217
Parkersburg, West Virginia 26102

Attention: Paul Bossert
Phone No.: (304) 863-4305

and

E. I. du Pont de Nemours and Company
Legal Department, Suite D-71
1007 Market Street
Wilmington, Delaware 19898

Attention: Bernard J. Reilly, Esq.
Phone No.: (302) 774-5445

XVII. AUTHORIZED SIGNATORIES/NON-ADMISSION.

The undersigned representatives state that they have had full and fair opportunity to review this Consent Order and have had opportunity to allow for their counsel to do the same, and therefore enter this Consent Order freely and with full knowledge of its terms and conditions.

The undersigned do hereby confirm that they have the authority to enter into this Consent Order and have the authority to bind their respective party.

Neither the terms of this Consent Order, nor execution thereof shall constitute an admission by DuPont of any fact or of any legal liability. DuPont expressly reserves all rights and defenses that may be available in any proceeding involving third parties or involving WVDEP and WVDHHR-BPH in any other matter.

This Consent Order may be signed in counterparts and shall be effective upon signature of all the Parties below ("Effective Date").

Entered this 14th day of NOVEMBER 2001, by:

WEST VIRGINIA DEPARTMENT OF ENVIRONMENTAL PROTECTION

BY:

William E. Adams

WILLIAM E. ADAMS, DEPUTY SECRETARY
West Virginia Department of Environmental Protection
1356 Hansford Street
Charleston, West Virginia 25301

Entered this 15th day of November, 2001, by:

WEST VIRGINIA DIVISION OF HEALTH AND HUMAN RESOURCES – BUREAU FOR PUBLIC HEALTH

BY:

DR. HENRY TAYLOR, COMMISSIONER
Bureau for Public Health
West Virginia Department of Health and Human Resources
Diamond Building, Room 702
350 Capitol Street
Charleston, West Virginia 25301

Entered this 15th day of Nov, 2001, by:

E. I. DU PONT DE NEMOURS AND COMPANY

BY:

PAUL BOSSERT, PLANT MANAGER

Attachment A

C8 GROUNDWATER INVESTIGATION STEERING TEAM

A team of scientists shall be assembled to assess the presence and extent of C8 in drinking water, groundwater and surface water at and around the DuPont Washington Works facility, and the Local, Letart, and Dry Run Landfills. The Groundwater Investigation Steering Team (GIST) shall include scientists from WVDEP, WVDHHR-BPH, EPA Region III, and DuPont. DuPont shall fund the GIST via an escrow account as provided in Section VIII of the attached Consent Order ("the Consent Order"). Disbursements from this account shall be authorized jointly by the WVDEP GIST leader, and the DuPont representative, Andrew S. Hartten.

A schedule summarizing key GIST tasks, submittals, start and end dates is provided at the end of this document.

GIST Member Organizations/Representatives/General Functions

WVDEP

David Watkins –Groundwater Protection- GIST team leader; escrow funds disbursement oversight; project management and coordination
George Dasher-advisor and technical review
Dee Ann Staats, Ph.D.-advisor

EPA Region III

Garth Connor-science advisor
Jack C. Hwang – Hydrogeologist
Roger Rheinhart-Environmental Engineer

DuPont

Andrew Hartten-Principal Project Leader/Hydrogeologist-technical review, project management and coordination of field investigation activities; escrow funds disbursement oversight.

WVDHHR-BPH

William Toomey-Manager, Source Water Assessment Program- Bureau for Public Health advisor

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GIST Team Objectives and Efforts

The primary objective of the GIST is to efficiently review and direct groundwater and surface water monitoring and investigation activities as prescribed in the Consent Order and in this Attachment. The GIST will utilize a phased approach and employ rapid team decision making toward meeting the requirements in an efficient and timely manner. Unless otherwise directed by the GIST, the tasks outlined below shall be performed by DuPont or its representatives.

The GIST will issue a final report(s) with findings and conclusions regarding groundwater quality in and around the Facilities, and the extent of groundwater contamination in and around the Facilities. The GIST final report shall further make recommendations regarding the need for any further work or actions that need to be taken to assure protection of groundwater quality and human health into the future.

The tasks set forth below and in the Consent Order are the minimum tasks to be performed by DuPont and the GIST pursuant to the Consent Order. Additional tasks may be necessary to assure the goals [full groundwater assessment and C8 impact, plume identification, and receptor identification] of the GIST and the Consent Order are met. Those tasks shall be agreed upon by the GIST.

Key Tasks of GIST

Task A: Groundwater Use and Well Survey/Groundwater Monitoring

- Objectives: Conduct a distance-phased groundwater well and water use survey within a 1-mile (and possibly 2 and 3-mile) radial distance or directionally focused distance of the Washington Works and Local, Letart, and Dry Run Landfills.[1]
- Summary: The phased approach to the water and groundwater well use survey will allow the GIST to focus efforts along established C8 impact transport pathways and cease activities in directions where impacts are not present or where there are minimal concentrations. Data results tables will be generated in a timely manner to allow the GIST to meet, evaluate the data, and determine the next course of action. The GIST will determine when the final groundwater well use survey shall be released.

  DuPont agrees to perform, under the supervision of the GIST and through an agreed-to third party, a groundwater use and well survey identifying and sampling all groundwater wells within a 1-mile radius of the three landfills set forth above and the Washington Works facility. The phased approach may be amended by the GIST should field conditions require, e.g., lack of sampling wells in the 1-mile radius, lack of quality sampling points within the 1-mile radius.

  Sampling shall be performed with the specific purpose of finding and measuring the C8 concentration in water. Should concentrations of C8 found in groundwater wells exceed 1 µg/l within the 1-mile radius, the GIST will determine

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1 The water use survey should be in substantially the same format as Attachment B.

whether to expand the well survey to a 2-mile radius, a 3-mile radius, or in a specific direction only. Drinking water wells that measure above 1 µg/l shall be re-sampled at a frequency to be determined by the GIST.

Note: The level of 1 ug/l is utilized in this Consent Order for monitoring purposes only and not as a benchmark for determining risk and this level may be adjusted as determined the GIST in furtherance of the tasks and objectives set forth in this Attachment.

- Timing: The initial well survey within a 1-mile radius of the Facilities will be conducted within 60 days of the Consent Order's Effective Date. Additional well survey activities will be conducted on a schedule to be determined by the GIST.

Task B: Assessment of Existing Groundwater and Surface Water Monitoring Data

- Objectives: Develop and implement a monitoring plan that determines the presence and extent of C8 in drinking water, groundwater, and surface water in and around the Washington Works facility and Local, Letart, and Dry Run Landfills and provide a compilation of all available groundwater/surface water monitoring and hydrogeologic characterization data for each facility, as reflected in Table A-1.
- Summary: The GIST will be tasked with an expedited evaluation of existing historical data and hydrogeologic information in order to prioritize the initial scope of work for continuing groundwater monitoring and any additional investigation activities (e.g., monitoring well installations) required under plume identification. DuPont shall provide all historical data and hydrogeologic information it may have related to the Facilities.
- Timing: Within 30 days of the completion of Task A, the GIST will review all the C8 analytical and facility hydrogeologic information to determine the scope of work for groundwater monitoring and additional investigation. The GIST will then establish a schedule for those activities. It is anticipated that a summary of all historical information for each facility will be submitted to GIST within 60 days of the Consent Order's effective date.

Task C: Plume Identification/Groundwater Assessment

- Objective: Determine the vertical and horizontal extent of any and all C8 impacted groundwater exceeding 1 ug/l or as directed by the GIST, which may determine a lower threshold than 1 ug/l. This task shall also include an assessment of C8 impacted groundwater at Letart Landfill and its impact on the Ohio River and public water supplies along the river.
- Summary: The GIST shall first review historical data and results of Task A to determine an appropriate scope of work. Activities should be prioritized to address groundwater plumes contributing to or with the potential to flow toward off-site receptors, with emphasis on those areas where groundwater is used as a drinking water source.

Upon completion of investigation activities, DuPont shall provide the GIST with predicted groundwater flow and contaminant transport models to assess future plume migration.

- Timing: Upon review of all available information and on a schedule to be determined by the GIST, the GIST will complete an initial evaluation of data to determine and prioritize plume identification.

The timing of the initial phase of plume identification/investigation activities and other activities will be on a schedule established by the GIST. Further investigatory activities needed and agreed to by the GIST to carry out the goals of the GIST shall be performed by DuPont on a schedule established by the GIST.

Modeling

Any and all modeling performed pursuant to this attachment and the Consent Order shall use Groundwater Modeling System, or some other model as approved by the GIST.

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TABLE A-1

COMPILATION OF HISTORICAL DATA AND MONITORING PLAN	
a. Dependent upon the availability of certain information, an historical data summary documented in a report that includes:	• A location map. • A site map showing the location of all known groundwater monitoring wells, residential groundwater wells and public water supply within a 1-mile radius the Facilities. • Top-of-groundwater maps. These should span the entire sampling life of the site and should be no less than yearly. If DuPont has only one year's worth of data for a given site, then these maps should be for each quarter; if DuPont has several years worth of data for each site, then these maps can be annual. • C8 concentration contour maps. These should span the entire sampling life of the site and should be no less than yearly. If DuPont has only one year's worth of data for a given site, then these maps should be for each quarter; if DuPont has several years worth of data for each site, then these maps can be annual. • All the C8 groundwater data that has been collected to date. These data should be submitted in easy-to-read tables. These tables should use the method, "<x", to designate all concentrations below the laboratory's minimum detection limit (not "ND" or some other abbreviation), and they should use "mg/" or "µg/" as the unit designation. • If unable to provide the above data, DuPont shall document the reasons why it is unable to gather and submit the information.
b. A groundwater monitoring plan for the Facilities which should address, at a minimum:	• C8 sampling. The samples should be taken from all the wells at the three landfill sites and from a select number of wells at the Washington Works plant. These select wells are to be chosen by the GIST before the groundwater monitoring program begins based on evaluation of historical data/information. The frequency of sampling shall be monthly for the first four months following the Effective Date and quarterly thereafter. Any new wells required for monitoring or plume identification purposes will be integrated in each site's groundwater monitoring program on a schedule agreed to by the GIST.

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	• Report of Results. Reporting should be quarterly and to the .WVDEP Groundwater Program at the following address. WVDEP Division of Water Resources Groundwater Program 1201 Greenbrier Street Charleston, West Virginia 25311 Re: DuPont/C8 monitoring. • Each report should include the following: (a) A site location map. (b) A site map showing the groundwater monitoring well locations. (c) A top-of-groundwater map. (d) A C8 concentration map. (e) Groundwater elevation and well screen data. (f) A table of all the historical C8 sampling data. Note: where available information allows, abbreviations should not be used to designate No Detect concentrations and the units "ppb" and "ppm" should not be used. (g) Laboratory analysis sheets. (h) Chain of custody records.

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Attachment B

GROUNDWATER WELL USE SURVEY

Name: _____

Address: _____
_____
_____

Phone: _____

Best Time to Contact Owner: _____

1. Do you have one or more water well(s) on this property? (It need not be in use currently.) If no, stop now and return survey. Yes _____ No _____

   County Water Well Permit No. _____

2. Is the well(s) currently (circle one) used unused or filled in?

3. Is the well(s) used for drinking water? Yes _____ No _____

4. Is this well(s) used for other purposes? If yes, please specify uses below:

_____
_____
_____
_____

5. What is the approximate frequency of use? Circle One:

   Daily Weekly Monthly Summer

6. Date last used? _____

7. Is there a pump in the well? Yes _____ No _____

8. Is there a conditioner, softener, chlorinator, filter, or other form of treatment for the system? Yes _____ No _____

   If so, what is the form of treatment? _____

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9. Is there any faucet where water does not first pass through the treatment system?
Yes _____ No _____

If yes, is it (circle one) inside or outside?

10. What year was the well constructed? _____

11. Please provide the following information regarding the well(s) if known: (circle one)

A. Total Depth (feet below ground surface):

30-60 60-90 90-120 120 or more

B. Casing Type:

PVC steel stone none other _____

C. Well Construction:

dug drilled open or uncased bedrock

D. Screened Interval (length in feet):

0-10 10-20 20-30 30-60 60 or more

E. Well Diameter (inches):

0-6 6-12 12-24 24 or more

Attachment C

C8 ASSESSMENT OF TOXICITY TEAM

A team of scientists shall be assembled to assess the toxicity and risk to human health and the environment associated with exposure to ammonium perfluorooctanoate (C8) releases from DuPont's activities. The C8 Assessment of Toxicity Team (CAT Team) shall include scientists from academia, government, non-profit organizations, and industry. The CAT Team also shall include the WVDEP Environmental Advocate, Pam Nixon, as a representative of West Virginia's citizens.

The WVDEP, utilizing funds from an escrow account funded by DuPont, shall contract with a non-profit organization, the National Institute for Chemical Studies (NICS), for the services described herein. Point of contact for the NICS shall be Jan Taylor, Ph.D. The NICS shall subcontract with Marshall University's Center for Rural and Environmental Health for services in risk communication provided by James Becker, M.D. and his staff. Dr. Becker shall familiarize himself with the toxicity of C8, the work performed by TERA as described herein, and attend public meetings to provide expertise in risk communication. The NICS shall subcontract with the non-profit scientific organization, Toxicology Excellence for Risk Assessment (TERA) whose point of contact is Joan Dollarhide, Ph.D. The TERA shall provide services in toxicology and risk assessment. Work assignments, tasks, and deliverables are described below.

CAT Team Member Organizations/ Representatives[1]/ General Functions

WVDEP

Dee Ann Staats, Ph.D. - Science Advisor - team leader; escrow funds disbursement oversight; project management and coordination; toxicology/risk assessment and communication;

Pam Nixon - Environmental Advocate - advisor;

NICS

Jan Taylor, Ph.D. –contractor administrative oversight;

James Becker, M.D. (Marshall University) - consultant in risk communication;

TERA (point of contact: Joan Dollarhide, Ph.D.)- consultant in toxicology/risk assessment;

[1] The parties may, in their discretion, elect to substitute their representatives with persons of similar qualifications.

DuPont

Gerald Kennedy, Director of Applied Toxicology and Health, Haskell Laboratory - reviewer toxicology; escrow funds disbursement oversight;

John Whysner, M.D. – toxicology/risk assessment and communications;

Paul Bossert – Washington Works Plant Manager – communications;

The following members of the CAT Team shall act as reviewers or advisors.

WV Department of Health and Human Resources – Bureau for Public Health (WVDHHR-BPH)

William Toomey – Manager, Source Water Assessment Program - advisor;
Barbara Taylor – Director, Office of Environmental Health Services - advisor;
Local representative - advisor;

Environmental Protection Agency (EPA)

Headquarters - Jennifer Seed – reviewer and advisor toxicology;
Region III Philadelphia -
Samuel Rotenberg, Ph.D. – reviewer and advisor toxicology/ risk assessment;
Garth Connor – advisor hydrogeology;
Roger Reinhart – reviewer and advisor Safe Drinking Water Act;
Cincinnati - John Cicmanec, DVM – reviewer and advisor toxicology;

Agency for Toxic Substances and Disease Registry (ATSDR)

Atlanta - John Wheeler, Ph.D. - reviewer and advisor in toxicology/ risk assessment;
Philadelphia - Lora Werner - coordinator for ATSDR;

Non-CAT Team Efforts

Other efforts are currently underway which may produce information for the CAT Team to utilize. The CAT Team will coordinate and communicate closely with these other efforts. These include:

1. Dupont's air modeling of C8 emissions from the Washington Works plant;

2. WVDEP's air modeling of C8 emissions from the Washington Works plant;

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First Public Meeting, CAT Team members shall familiarize themselves with the available toxicological information concerning C8.

A CAT Team meeting shall be held immediately prior to the first public meeting to: (1) conduct a site visit to the three landfills and the Washington Works Plant, and surrounding residential areas; (2) discuss the toxicity of C8 and other pertinent data; (3) prepare an agenda for the public meeting; (4) coordinate and prepare for the public meeting. Finally, the First Public Meeting will be held and public questions and comments will be recorded by WVDEP.

TABLE 1. TASKS OF CAT TEAM
Task A: Public Meetings (two meetings are anticipated) **Objective:** to inform the local citizens of the following: (in Meeting #1) intent to perform a groundwater well use survey and analysis for C8; intent to develop Screening Levels; and to ask for their cooperation in conducting the water use survey; and (in Meeting #2) results of survey, chemical analysis, and risk assessment. Note that an interim public meeting may be required should six months pass from the first public meeting and the CAT Team Final Report has not been issued. **Primary Responsibility:** Staats
Task B: Development of Provisional Reference Doses **Objective:** to develop Provisional References Doses for C8 for the inhalation and ingestion (and dermal, if possible) routes of exposure. **Primary Responsibility:** TERA
Task C: Development of Screening Levels Based on Protection of Human Health **Objective:** to utilize the Provisional Reference Doses to develop human health risk-based Screening Levels for C8 in air, water, and soil. Note a determination of the potential carcinogenicity of C8 will be conducted as well. **Primary Responsibility:** TERA
Task D: Ecological Data Review **Objective:** to review available information to determine whether sufficient studies have been performed and data have been collected to develop screening criteria for ecological receptors. **Primary Responsibility:** TERA
Task E: Draft Report and Final Report **Objective:** to present and discuss the results of the above tasks. **Primary Responsibility:** TERA

Phase II Tasks B, C, D, and E Development of Provisional Reference Doses and Screening Levels, and Risk Assessment

In Phase II, TERA shall conduct the toxicological and risk assessment activities. After having reviewed the toxicological information regarding C8 provided by WVDEP, TERA shall consult with toxicologists on the CAT Team, as coordinated by Dr. Staats, regarding its proposed approach for this project. Following such consultation, TERA

3. USEPA Draft Hazard Assessment which summarizes the available toxicity information regarding C8, to the extent completed prior to the assessment contemplated herein;

4. ATSDR's Health Consultation that estimates the risk to the community associated with C8 in drinking water from the Lubeck Public Service District, to the extent completed prior to the assessment contemplated herein.

5. Existing C8 concentrations in Lubeck Public Service District data.

6. Groundwater C8 Analysis (see GIST activities described in Attachment A) and Well Use Survey (see example survey in Attachment B) at the residences in the area of the 3 landfills and the Washington Works Plant.

Tasks of CAT Team

The tasks to be performed by the CAT Team are described briefly in Table 1, and in more detail below. These tasks are discussed below within the context of a Scope of Work for both Dr. Becker and for TERA as well.

Tasks of the CAT Team shall be organized into three phases. Phase I includes those tasks necessary to prepare for and hold the first public meeting. In Phase II, TERA shall conduct such scientific tasks as: reviewing available toxicity and epidemiological studies; developing Provisional Reference Doses and Screening Levels for protection of human health; evaluating existing information relative to ecological health; and conducting one general risk assessment involving comparisons of exposure concentrations to Screening Levels, for the three landfills and the Washington Works Plant, and the Lubeck Public Service District. TERA shall prepare a report on their findings. Phase III includes those tasks necessary to prepare for and hold the second public meeting. The results of the C8 groundwater analysis and risk assessment shall be presented in the second public meeting.

No communication between Dupont representatives and NICS, Dr. Becker, or TERA shall be permitted without the participation of Dr. Staats. All information will be provided to Dr. Becker and TERA by WVDEP; thus, all information contributed to the effort by Dupont shall be sent in triplicate to Dr. Staats for forwarding to Dr. Becker and TERA.

Phase I TASK A-1: First Public Meeting

Two public meetings are anticipated for this project. The First Public Meeting shall occur in Phase I for the purposes of introducing the CAT Team and other involved parties to the public; relating historical information on previous concentrations of C8 in Lubeck Public Service District water supply; informing the citizens of the ensuing activities; and inviting the public to participate by cooperating with sampling and survey efforts in the Groundwater C8 Analysis and Well Use Survey. In order to prepare for the

shall develop Provisional Reference Doses for C8 for the oral, inhalation, and dermal (if possible) routes of exposure. Then TERA shall calculate Screening Levels for water, soil and air based on the risk factors they have estimated. TERA shall perform one general risk assessment involving comparison of exposure concentrations to Screening Levels for the three landfills and the Washington Works Plant, and the Lubeck Public Service District water supply, that focuses on current risk to human health, including workers and residents. This risk assessment shall include: (1) identification of reasonably anticipated land use, surface water and groundwater use; (2) identification of receptors; (3) identification of exposure pathways; (4) identification of exposure concentrations; and (5) comparison of exposure concentrations to appropriate Screening Levels. TERA shall utilize data obtained from the other efforts discussed above such as air modeling; groundwater C8 concentrations in residential and public wells; residential groundwater well use survey; the USEPA's Draft Hazard Assessment; and ATSDR's Health Consultation (if available). TERA also shall review available information to determine whether sufficient studies have been performed and data have been collected to develop screening criteria for protection of ecological health, particularly aquatic life. TERA shall prepare a draft and a final document that discusses the results of their efforts and summarizes the data utilized from other efforts. As the tasks of the CAT Team and other involved parties' progress, data gaps and research recommendations may become evident. These shall be included in TERA's report as suggestions for further research to elucidate the toxicity of C8.

Phase III Second Public Meeting

The purpose of the Second Public Meeting is to present to the citizenry the results of the efforts of the GIST and CAT Teams including C8 concentrations in groundwater from residential wells and public wells the screening levels and the general risk assessment. Air modeling results of the efforts of WVDEP and Dupont will be discussed also. The WVDEP will address any further actions that may be necessary.

SCOPE OF WORK FOR
JAMES BECKER, M.D.

Dr. Becker is a medical doctor specializing in environmental health at the Marshall University School of Medicine Center for Rural and Environmental Health. He will be assisting the WVDEP in his specialty area of risk communication at the two anticipated public meetings. The specific tasks assigned to Dr. Becker are described below.

Phase I Task A-1: First Public Meeting

Dr Becker will assist in preparation for the first public meeting, and attend the meeting providing expertise in risk communication . He will familiarize himself with the available toxicological data, which will be provided to him by WVDEP, with particular emphasis on the epidemiological studies. Note that the toxicological data already has been summarized in the Draft Hazard Assessment prepared by USEPA. No literature search or document retrieval will be required. Specific subtasks required in Phase I to prepare for the first public meeting are described below:

Subtask 1 – Familiarization with toxicological data provided by WVDEP including but not limited to:

a. 8 compact discs of information provided to USEPA under TSCA by 3M Corp (note only a small portion of this information concerns C8);
b. Draft Hazard Assessment document from USEPA;
c. ACGIH Threshold Limit Value (TLV).
d. Journal articles and other information provided by WVDEP.

Subtask 2 – Attend a meeting prior to the first public meeting to:

a. conduct a site visit of the 3 landfills and the Washington Works Plant, and local residential areas;
b. discuss and prepare an agenda;
c. discuss the toxicology and risks associated with C8 with the other CAT Team members.

Subtask 3 – Attend First Public Meeting

Phase III Task A-2 Second Public Meeting

Dr Becker will assist in preparation for the second public meeting, and attend the meeting providing expertise in risk communication. The following subtasks will be required:

Subtask 1 – Familiarization with the toxicological and risk assessment report prepared by TERA;

Subtask 2 – Attend a meeting prior to the second public meeting to:

a. discuss the toxicology and risks associated with C8 with the other CAT Team members;
b. discuss and prepare an agenda.

Subtask 3 – Attend Second Public Meeting

Note that the second public meeting is assumed to be the final public meeting; however, results of data collection may warrant additional public meetings and an expansion of the Scope of Work.

SCOPE OF WORK FOR TERA

TERA (Toxicology Excellence for Risk Assessment) is a non-profit organization that applies sound toxicological data to the risk assessment process to find common ground between environmental, industry, and government groups. TERA will be providing services in toxicology and risk assessment. TERA scientists will be developing risk factors and screening criteria; and conducting one general risk assessment for the 3 landfills, Lubeck Public Service District water supply and the Washington Works Plant. The specific tasks assigned to TERA are described below.

Phase II Tasks B, C, D, and E: Development of Provisional Reference Doses and Screening Levels, and General Assessment of Risk

Subtask 1 – TERA staff will familiarize themselves with the toxicological data provided to by WVDEP. No literature search or document retrieval will be required. Toxicological data to be provided to TERA shall include but is not limited to the following:

a. 8 compact discs of information provided to USEPA under TSCA by 3M Corp (note only a small portion of this information concerns C8);
b. USEPA Draft Hazard Assessment for C8;
c. Journal articles and other information submitted to WVDEP by DuPont.

Subtask 2 – TERA staff will:

a. identify all possible critical toxicological studies suitable for developing Reference Doses for the oral, inhalation, and dermal (if possible) routes of exposure;
b. outline methodology for developing said Reference Doses and for developing Screening Levels for air, water, and soil based on said Reference Doses corresponding to each critical study identified in subtask 2-a;
c. convene a meeting at the TERA facility in Cincinnati, Ohio, to present their findings in subtask 2-a and 2-b, and consult with CAT Team toxicologists as coordinated by Dr. Staats;
d. finalize Reference Doses and Screening Levels based on recommendations of the CAT Team toxicologists as coordinated by Dr. Staats.

Subtask 3 – TERA shall conduct one general risk assessment for the three landfills and Washington Works Plant, and the Lubeck Public Service District water supply based on current risk to human health. This risk assessment shall include:

a) identification of reasonably anticipated land use, surface water and groundwater uses;

b) identification of receptors;
c) identification of exposure pathways;
d) identification of exposure concentrations;
e) comparison of exposure concentrations to appropriate Screening Levels;

TERA shall utilize the following data in the risk assessment process:

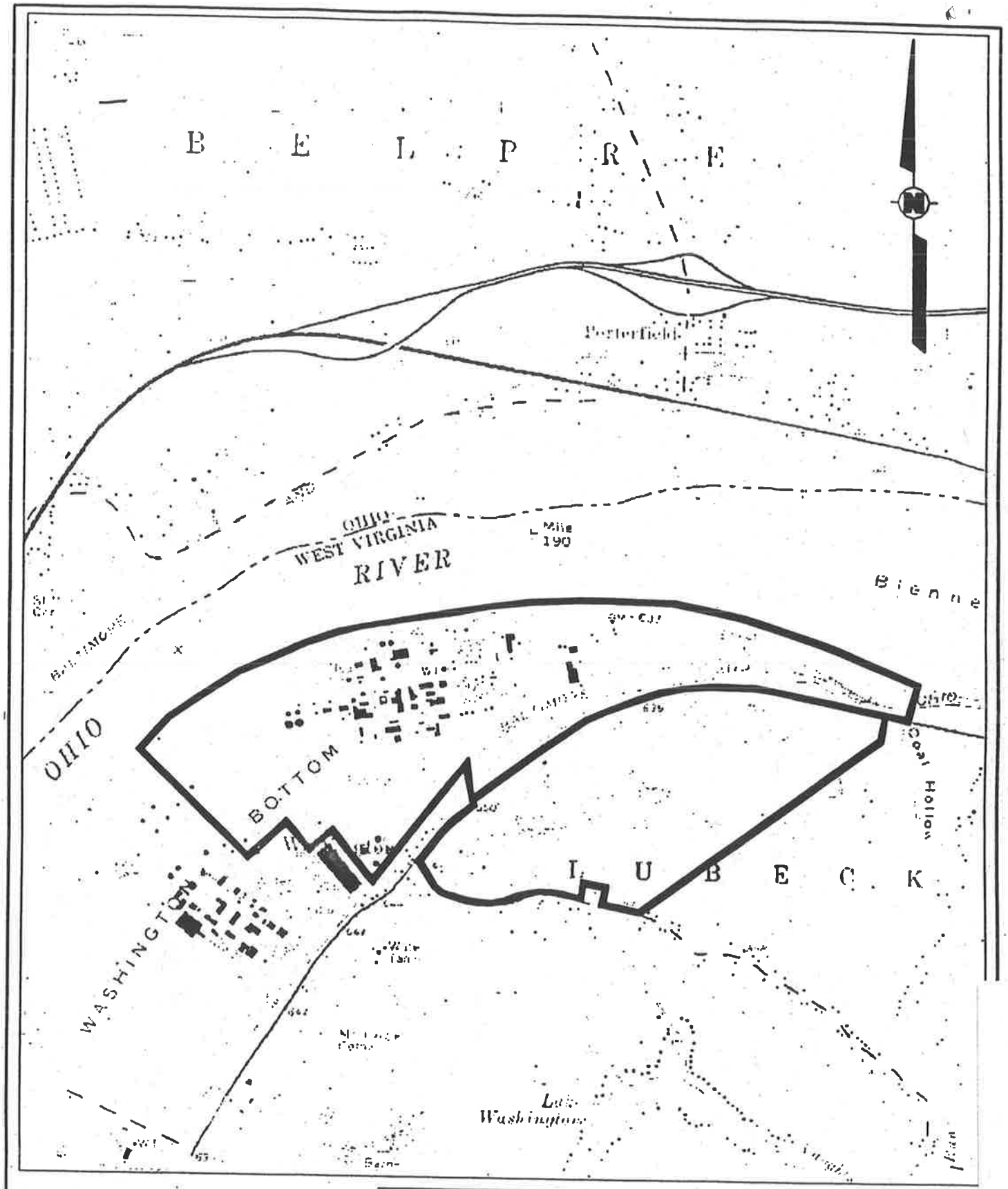
a) air modeling data from DuPont;
b) air modeling data from WVDEP;
c) water use data from the Well Use Survey;
d) groundwater data from the Groundwater Well Analysis of C8 for residential wells;
e) drinking water data from Lubeck Public Service District wells;
f) any available ATSDR Health Consultation that assesses potential health effects from exposure to C8 in public supply drinking water.

Subtask 4 – TERA shall review the ecological data and determine whether there is sufficient information to support the development of a C8 Screening Level for protection of ecological health

Subtask 5 – TERA shall compile and discuss the results of the above tasks into a comprehensive report (draft and final versions), which also refers to and provides a brief summary of the following:

a) USEPA's Draft Hazard Assessment of C8;
b) DuPont's air modeling data;
c) WVDEP's air modeling data;
d) groundwater data from the Groundwater C8 Analysis and Well Use Survey of Local Residents, and Lubeck Public Service District;
e) ATSDR Health Consultation that assesses potential health effects from exposure to C8 in public supply drinking water, if available.

Additionally, TERA shall include in the report any insights or recommendations for future research gleaned during this process that would further elucidate the toxicity of C8. Also, TERA shall provide in the report of a summary discussion of the relevance the carcinogenicity of C8 in rats to humans.



Source: USGS Little Hocking, Ohio – Quadrangle

Corporate Remediation Group
An Alliance between DuPont and URS Diamond

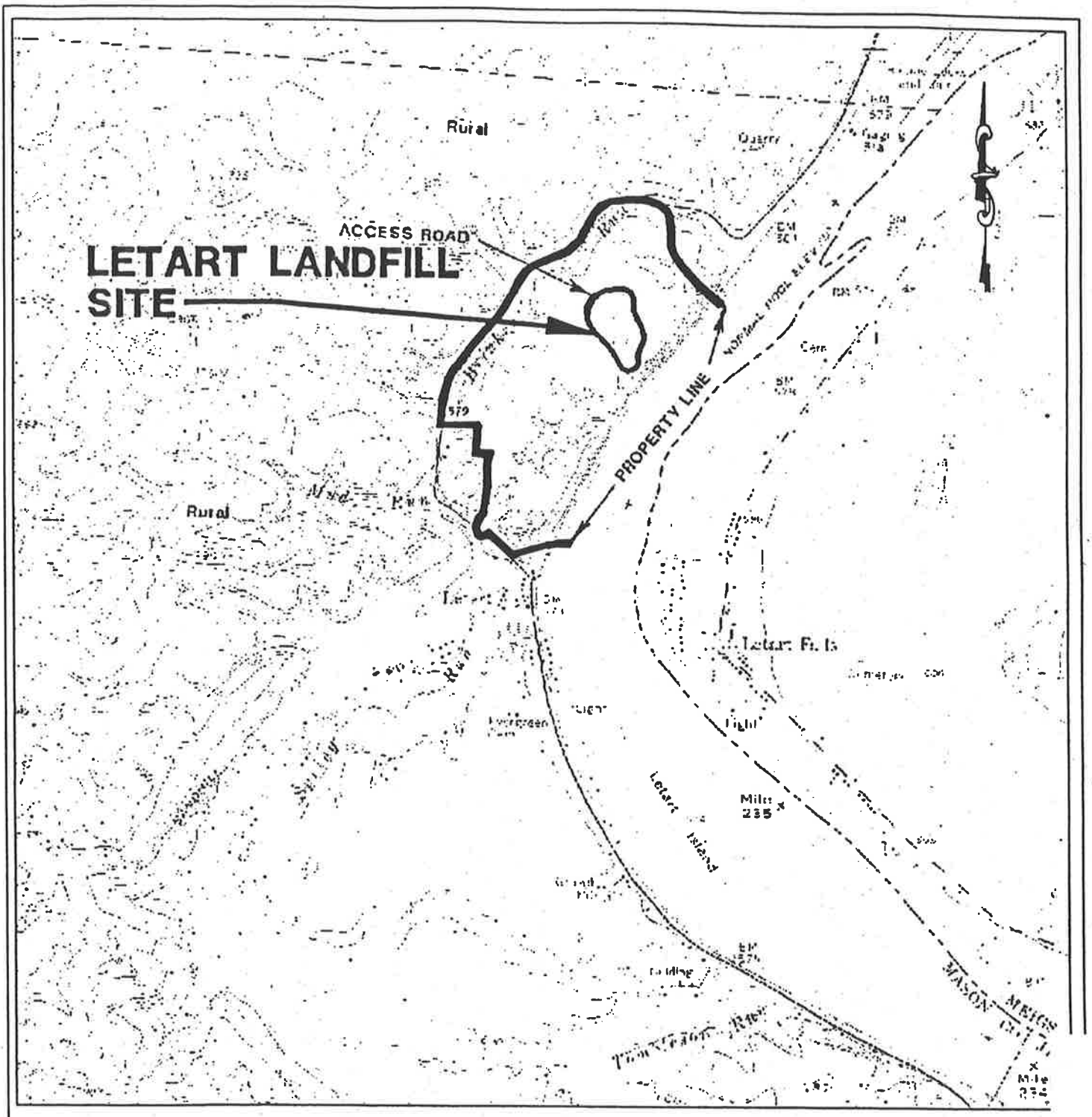
EXHIBIT 1

SITE LOCATION MAP

DuPont Washington Works
Washington, West Virginia

SCALE: Not to scale	DESIGNED: DEL	DRAWN: DEL	CAD FILE NO: SITE_LOC
DATE: 8/27/01	CHECKED: M. HOLLIDAY	APPROVED:	FIGURE: 1

EPA 01426



SOURCE: NEW HAVEN, WV-OHIO QUADRANG

Corporate Remediation Group
An Alliance between DuPont and The E-C Diamond Group

SITE LOCATION MAP

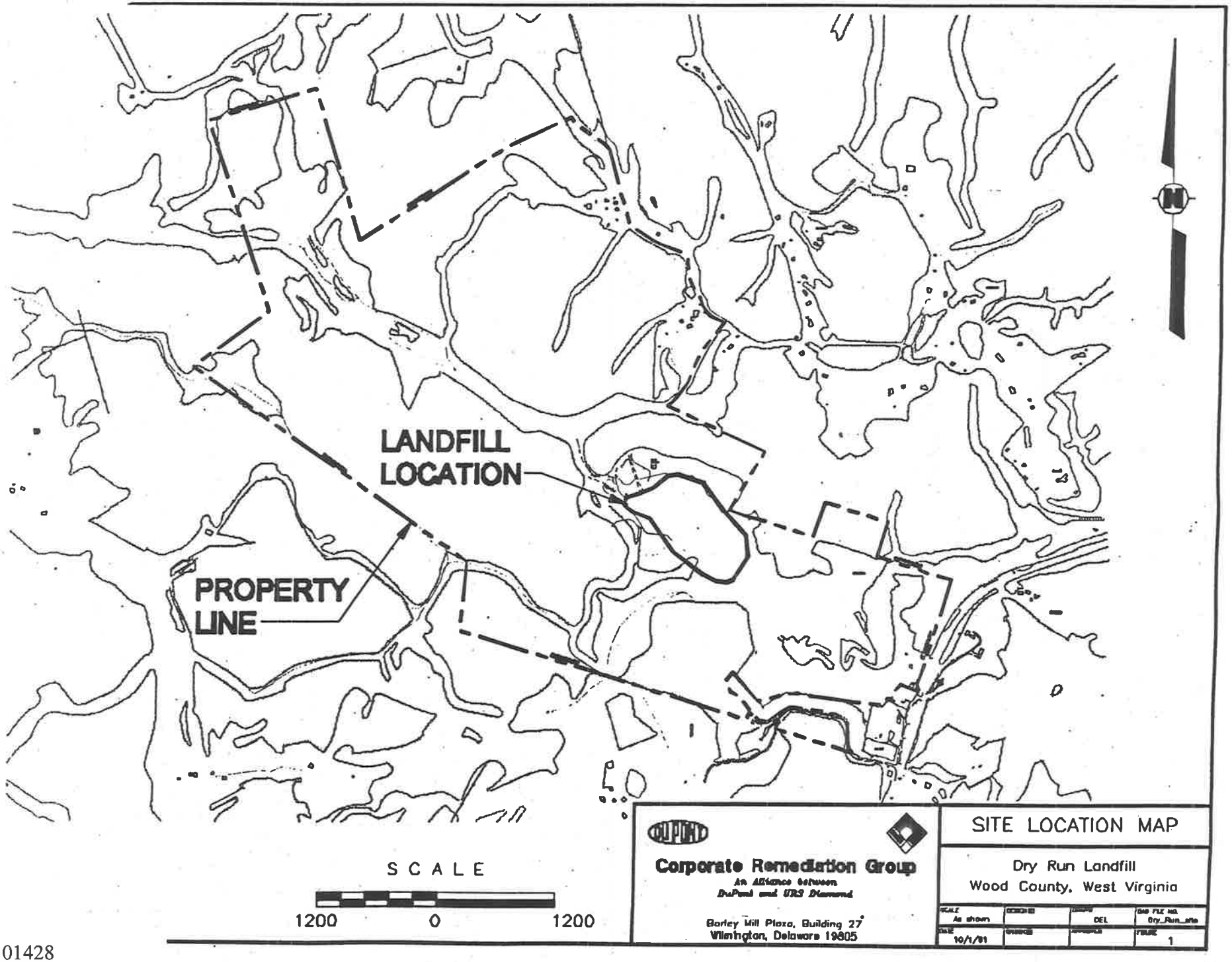
Letart Landfill Site
Parkerburg, WV

SCALE	DESIGNED	DRAWN	CAD FILE NO.
As shown	TDL	DEL	7264A003
DATE	CHECKED	APPROVED	ISSUE
12/21/9			1

EXHIBIT 2

EPA 01427

EXHIBIT 3



EPA 01428

DOCUMENT 55

Anal. Chem. **2001**, *73*, 2200−2206

Determination of Perfluorinated Surfactants in Surface Water Samples by Two Independent Analytical Techniques: Liquid Chromatography/ Tandem Mass Spectrometry and ^{19}F NMR

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Perfluorinated surfactants are an important class of specialty chemicals that have received recent attention as a result of their persistence in the environment. Two analytical methods for the determination of perfluorinated surfactants in aqueous samples were developed in order to investigate a spill of 22 000 L of fire retardant foam containing perfluorinated surfactants into Etobicoke Creek (Toronto, Ontario). With the first method, aliquots of surface water (0.2−200 mL) were preconcentrated using solid-phase extraction. Liquid chromatography/tandem mass spectrometry was employed for identification and quantification of each perfluorinated surfactant. Total perfluorinated surfactant concentrations in surface water samples ranged from 0.011 to 2270 μg/L, and perfluorooctanesulfonate was the predominant surfactant observed. Interestingly, perfluorooctanoate was detected in surface water sampled upstream of the spill. A second method employing ^{19}F NMR was developed for the determination of total perfluorinated surfactant concentrations in aqueous samples (2−100 mL). By ^{19}F NMR, the surface water concentrations ranged from nondetect (method detection limit, 10 μg/L for a 100-mL sample) to 17 000 μg/L. These methods permit comprehensive evaluation of aqueous samples for the presence of perfluorinated surfactants and have applicability to other sample matrixes.

Perfluorinated surfactants are a specialty class of chemicals employed for a variety of applications, including lubricants, paints, cosmetics, and fire-fighting foams.[1−3] In general, perfluorinated surfactants are classified into one of four categories: anionic, cationic, nonionic, and amphoteric, with anionic surfactants being the most significant class.[2] Perfluorooctanesulfonate (PFOS) is an important perfluorinated surfactant as well as a precursor to other perfluorinated surfactants.[4] Perfluoroalkanesulfonate salts and perfluorocarboxylates are present in fire-fighting foam formulations, including aqueous film forming foams (AFFFs).[5,6] In May 2000, the 3M Company (St. Paul, MN) announced that it would discontinue the production of two anionic perfluorinated surfactants, PFOS and perfluorooctanoic acid (PFOA), in part because of the compounds' persistence in the environment.[7−10] The company's decision to reduce production of commercial products was partially based upon the detection of PFOS concentrations (10−100 μg/L) in blood samples from the United States, Japan, Europe, and China[8,11] and biota samples.[7]

Historically, mineralization techniques[2,12−15] were used for the determination of total organofluorine compounds in environmental and biological samples. For example, in the 1960s, Taves[16,17] reported the detection of organic fluorine compounds in human blood. The total organofluorine technique is not specific in multifluorinated surfactant systems. In the case of fire-fighting foams as well as other industrial applications, several perfluorinated surfactants are potentially present and the use of the total organofluorine technique would only provide nonspecific, semiquantitative information for the fluorochemicals present in the sample. Furthermore, the total organofluorine technique may not be rigorous enough to quantitatively determine those perfluori-

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† University of Toronto.
‡ University of Guelph.
§ National Water Research Institute.

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10.1021/ac0100648 CCC: $20.00
Published on Web 04/13/2001

nated surfactants, such as perfluoroalkanesulfonic and perfluorocarboxylic acids, that are thermally stable.[2] Another nonspecific analytical method to determine the presence of anionic perfluorinated surfactants in environmental matrixes is the methylene blue active substances (MBAS) test.[18,19] The application of the MBAS test to perfluorinated surfactants in environmental matrixes is limited, because the test does not allow for the identification of individual anionic surfactants.[5]

Because most perfluorinated surfactants lack chromophores, the compounds are not easily amendable to traditional high performance liquid chromatography (HPLC) methodologies. Ohya et al.[20] measured concentrations of perfluorinated carboxylic acids in biological samples by HPLC using fluorescence detection. The multiple steps of this method,[20] which include ion pairing and derivatization, limit its application to environmental samples.

Derivatization techniques coupled with gas chromatography with electron capture[21,22] and mass spectrometric[5,19,23,24] detection were employed for the determination of perfluorinated surfactants. Perfluorocarboxylates were determined in groundwater by derivatization to their methyl esters then gas chromatography/mass spectrometry (GC/MS);[5,19,24] however, derivatization techniques in combination with GC/MS have limited utility for the detection of perfluorinated surfactants in environmental matrixes.[5] For example, PFOS is nonvolatile, and its derivatives are unstable as a result of the excellent leaving group properties of the perfluoroalkanesulfonic group.[25,26]

In the past, ^{19}F NMR was employed for the determination of perfluorinated surfactants in biological samples. In the 1970s, Guy et al.[27] fractionated and concentrated a large volume of human blood to isolate fluoroorganic compounds, then subsequent analysis by ^{19}F NMR yielded a spectrum showing strong similarity to PFOA. Hagen et al.[22] employed ^{19}F NMR with perfluorinated compounds in rat serum; however, early applications of the technique were not necessarily quantitative.[2] More recently, ^{19}F NMR methods have been applied to fluorinated acetic acids[28] and pesticides for quantitative purposes. Because of the low sensitivity of NMR techniques, preconcentration procedures may be required for the determination of perfluorinated surfactants in environmental matrixes. Benefits of the ^{19}F NMR method for the determination of perfluorinated surfactants include sharp, well-resolved peaks, specificity to fluorine, accessibility to instrumentation, and relatively low maintenance expense. An additional benefit of ^{19}F NMR includes the lack of matrix interferences, which results in low signal-to-noise ratios; the absence of interferences is due in part to the fact that multifluorinated compounds are not naturally occurring.

It is desired that new analytical methodology be routine and robust and that it provide quantitative, structure-specific information for perfluorinated surfactants; few such methods are reported in the open literature.[4,11,19,29,30] Direct loop injection negative electrospray ionization mass spectrometry was employed for the determination of perfluoroalkanesulfonates in groundwater sampled from fire-training areas.[19] Olsen et al.[11] reported concentrations for PFOS in blood serum by liquid chromatography/thermospray mass spectrometry. Recently, concentrations of PFOS, perfluorohexanesulfonate (PFHxS), PFOA, and perfluorooctanesulfonylamide were reported in human serum samples by ion pair extraction coupled with liquid chromatography/tandem mass spectrometry (LC/MS/MS).[30] Analytical methods that include chromatographic separations of individual perfluorinated surfactants combined with mass spectrometric detection allow for the confirmation of each compound present in the environmental sample.

The accidental release of a multiple perfluorinated surfactant fire-fighting foam product into surface water highlights the need for analytical methods to determine perfluorinated surfactants at environmentally relevant concentrations. In this paper, we detail (1) a liquid chromatography/tandem mass spectrometry method for the determination of individual homologues of two classes of anionic perfluorinated compounds in aqueous samples, (2) a ^{19}F NMR method for the determination of total perfluorinated surfactant concentrations present in aqueous samples, and (3) the application of the two developed analytical methods to surface water samples collected after an accidental discharge of perfluorinated surfactants.

EXPERIMENTAL SECTION

Standards and Reagents. Standards of potassium perfluorooctanesulfonate (86.4%) and potassium perfluorohexanesulfonate (99.9%) were provided by the 3M Co. Standards of perfluorooctanoic acid (98%) and perfluorododecanoic acid (PFDoA, 95%) were purchased from Aldrich Chemical (Milwaukee, WI), and perfluorohexanoic acid (PFHxA) was obtained from Oakwood Research Chemicals (West Columbia, SC). Ammonium acetate (98%) was purchased from Aldrich Chemical Co. The ^{19}F NMR internal standard, 4′-(trifluoromethoxy)acetanalide (TFMAA). NMR solvent methyl-d_4 alcohol, and the relaxation agent, chromium acetylacetonate [$Cr(acac)_3$] were purchased from Aldrich Chemical Co. (Mississauga, Ontario, Canada). All reagents and solvents were used as received.

Spill Information and Sample Collection. On June 8, 2000, a fire alarm malfunctioned at an airline hanger at L. B. Pearson International Airport, Toronto, Ontario, Canada. The malfunction released 22 000 L of fire retardant foam and 450 000 L of water from the sprinkler system into storm sewers, which led to Spring Creek, then into Etobicoke Creek, which empties into Lake Ontario.[31]

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It has been reported that perfluorinated surfactants may adsorb to glass;[21] therefore, all of the surface water samples were collected in polypropylene bottles. Etobicoke Creek samples were collected over a period of 153 days after the AFFF spill. Sample site 1, which is upstream of the airport and the AFFF spill, was collected as background surface water. Samples were stored without preservation at 4 °C prior to analysis. Additionally, care was taken to avoid sample contact with Teflon, which is known to contain one or more of the analytes of interest.[24]

Surface Water Preconcentration. The surface water samples (0.2 to 200 mL) were allowed to warm to room temperature, shaken, and sub-sampled. To preconcentrate the surface water samples, the aqueous sample was passed through a preconditioned C18 (Supelclean ENVI-18, Supelco; Bellefonte, PA) cartridge (500 mg, 3 mL). The extraction medium was preconditioned by passing 5 mL of methanol through the C18 cartridge, followed by 5 mL of deionized water. The sample container was rinsed with 2 mL of deionized water, and the sides of the sample cartridge were rinsed with two 1-mL aliquots of deionized water. The C18 cartridges were centrifuged to remove any residual water and then were placed in clean, polypropylene centrifuge tubes. For the samples to be analyzed by LC/MS/MS, the analytes were eluted with 2 mL of methanol and again centrifuged; the extracts were refrigerated at 4 °C prior to analysis. The internal standard, PFDoA, was added to each methanol extract just prior to the analysis by LC/MS/MS. For ^{19}F NMR analysis, the analytes of interest were eluted from the columns using 1 mL of deuterated NMR solvent, then quantitatively transferred to a volumetric flask containing $Cr(acac)_3$ and the internal standard, TFMAA.

Liquid Chromatography/Tandem Mass Spectrometry and Quantitation. Standard compounds were infused through a syringe pump (KD Scientific; Boston, MA) at a flow rate of 25 μL/min for positioning of the ion sprayer and tuning of the mass spectrometer. The methanol extracts (25 μL injection volume) with the internal standard present were chromatographed [guard column (Security Guard, C18, Phenomenex; Torrance, CA) and Genesis C8 column, 2.1 mm × 50 mm, 4 μm, (Chromatographic Specialties; Brockville, Ontario)] using high performance liquid chromatography (model 700, Waters; Milford, MA) with a flow rate of 300 μL/min. The gradient was operated from 40 to 95% eluent B for 5 min, then held at 95% eluent B for 10 min, where eluent A is 10 mM aqueous ammonium acetate, and eluent B is 10 mM ammonium acetate in methanol. The total run time was 15 min, with an equilibration time of 10 min between successive samples.

All of the mass spectra were acquired on a Quattro LC liquid chromatography tandem mass spectrometer equipped with a Z-spray interface (Micromass; Manchester, U.K.) employing negative electrospray ionization. The capillary voltage was 2.7 kV, and the cone voltage ranged from 14 to 55 V, depending on the individual compound of interest (Table 1). The dwell time was 0.2 s. The source block and desolvation temperatures were 130 and 330 °C, respectively. The nebulizer and desolvation gas flowrates were 50 and 550 L/hr, respectively. During tandem mass spectrometric analysis, argon was used as the collision gas (5.0×10^{-3} mBar) where the collision energy (12 to 45 eV, see Table 1) was varied for optimal performance for each compound. Full-scan mode was employed for the identification of individual surfactants. For quantification, multiple reaction monitoring (MRM) was used for each perfluorinated surfactant (Table 1).

Table 1. Optimized LC/MS/MS Parameters for the Determination of Perfluoroalkanesulfonates and Perfluorocarboxylates in Aqueous Samples

compd	parent ion, m/z	daughter ion monitored, m/z	collision energy, eV	cone voltage, V	instrumental detection limit, pg
PFPeA	263	219	12	14	3
PFHxA	313	269	12	14	3
PFHpA	363	319	12	14	4
PFOA	413	369	12	14	1
PFDoA	613	569	14	18	8
PFBS	299	99	33	40	5
PFHxS	399	99	40	45	3
PFOS	499	99	45	55	4

Calibration curves constructed for PFOS and PFOA ranged from 0.85 to 208 μg/L and 0.46 to 325 μg/L, respectively, and were linear, with r^2 typically > 0.99. When quantification of PFHxS was performed, we assumed a response factor equal to an equimolar amount of PFOS.

Spike and Recovery of Surface Water Samples by LC/MS/MS. Spike and recovery experiments were performed to determine the precision and accuracy of the method. One set of spike and recovery experiments was performed using deionized water that had been previously determined to contain neither PFOS nor PFOA above detection. Six samples (50-mL) were spiked to three different final concentrations, 0.33, 0.83, and 1.66 μg/L of PFOS and 0.52, 1.30, and 2.60 μg/L of PFOA. With this experiment, the spiked deionized water samples were preconcentrated and analyzed by LC/MS/MS.

Standard addition analysis was performed using a surface water sample (sample 3-4, $n = 3$) that had been previously determined to contain PFOS and PFOA above detection. Known amounts of PFOS and PFOA were added to the samples. For example, surface water from sample 3-4, which contained a background concentration of 66.7 and 1.14 μg/L for PFOS and PFOA, respectively, was spiked with standards to give final concentrations of 133 and 105 μg/L of PFOS and PFOA, respectively.

^{19}F NMR Spectrometer Parameters and Quantitation. All of the ^{19}F NMR spectra were obtained on a Varian Unity 500, 3-channel spectrometer operating at 470.297 mHz at 26 °C. The NMR was equipped with a 5-mm Nalorac ^{19}F proton decoupling probe. To zero-fill the free induction decays (FID), the Fourier number was set to equal twice the number of data points. All of the chemical shifts were relative to $CFCl_3$ (0.000 ppm). The NMR spectra were acquired with optimized parameters for which the 90° pulse width was 10.5 s and the spectral window was −50 to −85 ppm.

Acquisition time was limited to 45 min (1000 transients), and control (blank) samples were set at 10 h (13 888 transients). The spin−lattice relaxation times (T_1) for PFHxA, PFOA, PFHxS, and PFOS in methanol with 4 mg $Cr(acac)_3$ were determined to be 0.3143, 0.3546, 0.3063, and 0.3886 s, respectively. The T_1 for PFOS without the addition of $Cr(acac)_3$ was 1.753 s. For quantification, the minimum recycling delay time (D_1) value (equal to approximately 5 × the T_1) was set to the maximum T_1 (PFOS, 0.3886 s) for the analytes of interest, 2.0 s. The measurements of T_1

enables the optimization of D_1 values, which then allows the maximum sensitivity for a given number of transients. The data were processed with an exponential multiplication corresponding to a 10 Hz signal width.

The total concentration of perfluorinated surfactants, which includes perfluoroalkanesulfonates and perfluorocarboxylates, was determined by a linear calibration curve ($r^2 > 0.99$) using known concentrations of PFOS (1.00−105 μg/mL) and the internal standard, TFMAA.

Spike and Recovery Experiments by ^{19}F NMR. To determine the extraction efficiency of the preconcentration solid-phase extraction step, triplicate field water samples (100-mL) were spiked with known concentrations of PFOS, 0.15 and 0.70 mg/L. A second experiment was conducted using a single field water sample (100-mL) that contained a mixture of PFOS, PFHxS, PFOA, and PFHxA, with concentrations of 25, 10, 10, and 10 mg/L, respectively.

RESULTS AND DISCUSSION

Optimization of the LC/MS/MS Method. Standards of PFOA and PFOS had retention times of 9.2 and 9.8 min, respectively, and the internal standard, PFDoA, eluted at 11.3 min. Preliminary experiments using a hydrocarbon surfactant, sodium 1-tetradecane sulfonate (Lancaster; Pelham, NH), as the internal standard were found to be unacceptable, because the hydrocarbon surfactant did not behave in a manner similar to the perfluorocarbon surfactant standards during electrospray ionization. Because of its perfluorinated characteristics, a higher chain perfluorocarboxylate homologue, PFDoA, was employed for the internal standard. Surface water samples were analyzed without the addition of PFDoA to ensure the absence of the compound prior to its use as an internal standard.

For future applications of this method, an alternative internal standard would be an isotopically labeled perfluoroalkanesulfonate and perfluorocarboxylate, which would behave almost identically to that of nonlabeled compounds during electrospray ionization. A second option for an internal standard is a partially fluorinated internal standard, such as 1*H*,1*H*,2*H*,2*H*-perfluorooctanesulfonate.[4,30]

Standard solutions of perfluoroalkanesulfonates and perfluorocarboxylates were used to optimize the collision energy and cone voltage for each individual compound (Table 1). Optimal collision energies and cone voltages were similar to those previously reported in the literature.[30] The parent ion ($[M]^-$) for individual perfluorinated surfactants was observed by negative electrospray ionization; for example, the parent ion for PFOS, *m/z* 499, was observed for a PFOS standard. The molecular ions observed for homologues of perfluoroalkanesulfonates and perfluorocarboxylates were separated by 50 amu, which corresponded to the CF_2 group present within the perfluorocarbon chain (Table 1).

Because of an increase in sensitivity in MRM mode over full-scan mode, MRM mode was employed for quantification. For example, the transition of PFOS, *m/z* 499 ($C_8F_{17}SO_3^-$), to *m/z* 99 (daughter fragment, FSO_3^-) was monitored (Table 1). The two predominant daughter ions produced from the fragmentation of *m/z* 499 with optimal parameters were *m/z* 80 (SO_3^-) and *m/z* 99 (FSO_3^-). Although the intensity of the daughter ion at *m/z* 80 is greater than that of the daughter ion at *m/z* 99, the latter ion was monitored for quantification because the presence of fluorine gives greater specificity for perfluorinated surfactants. For the perfluorocarboxylate compounds, the perfluorocarbon chain fragment was monitored (Table 1). As an example, for PFOA, the transition from the parent ion, *m/z* 413 ($C_7F_{15}COO^-$), to *m/z* 369 ($C_7F_{15}^-$) was monitored for quantification. This is in contrast to the methodology by Hansen et al.[30] that monitored *m/z* 169 for PFOA, which corresponds to ($C_3F_7^-$).

Because of the sorptive nature of perfluorinated compounds, particularly perfluoroalkanesulfonates, instrument cleanliness is extremely important. Throughout sample analyses, the cone and baffle were cleaned with methanol at least every 5−10 samples or as often as necessary. To guarantee data quality, standards were analyzed and interspersed throughout the sample runs to verify the calibration curve validity and instrument performance. To ensure that no laboratory contamination or instrumental carryover issues existed, 100-mL deionized water samples were routinely extracted and analyzed by LC/MS/MS. Additionally, reagent blanks (methanol) were run periodically throughout sample sets. It should be noted that when surface water samples were not preconcentrated and were directly injected onto the HPLC column, significant carryover was observed for repetitive highly concentrated samples. The carryover issues were eliminated when the analytes were in methanol rather than an aqueous matrix.

Accuracy, Precision, and Detection Limits for LC/MS/MS. Although it was our original intent to use the surface water collected upstream of the airport and spill (sampling site 1) for spike and recovery experiments, it was found to contain detectable concentrations of PFOA (0.011 and 0.028 μg/L). One possible explanation for the observed concentrations in surface water upstream of the discharge is widespread environmental contamination of perfluorocarboxylates. Our current research focuses on possible sources for PFOA and other perfluorinated surfactants in Etobicoke Creek.

The spike and recovery experiment was performed using 50-mL sample volumes and percent recoveries for PFOS (at three different concentration levels) ranged from 49 to 130%, with a mean of 68% ± 45%. The percent recoveries for PFOA (at three different concentration levels) ranged from 80 to 109%, with an average of 93% ± 11%.

Other spike and recovery experiments using smaller chain homologues of perfluoroalkanesulfonate and perfluorocarboxylates indicate that the extraction efficiency using C18 for the shorter chain homologues is <100%; for example, the percent recoveries for perfluorobutanesulfonate (PFBS) and perfluoropentanoate (PFPeA) were 35 and 15%, respectively. The applicability of this method is limited to environmental samples with 6−10 perfluorinated carbons. Clearly, additional work is needed in the future to improve extraction efficiencies employing solid-phase extraction for the perfluorinated compounds, particularly the perfluoroalkanesulfonates.

Because detectable concentrations of PFOS and PFOA were found in surface water samples, a standard addition experiment was performed to determine the recoveries of PFOS and PFOA. The percent recoveries of PFOS and PFOA spiked into surface water samples were calculated as the measured spiked concentration minus the original sample concentration divided by the concentration added to the sample multiplied by 100. The percent recoveries of PFOS for sample 3-4 ($n = 3$) was 49, 55, and 72%

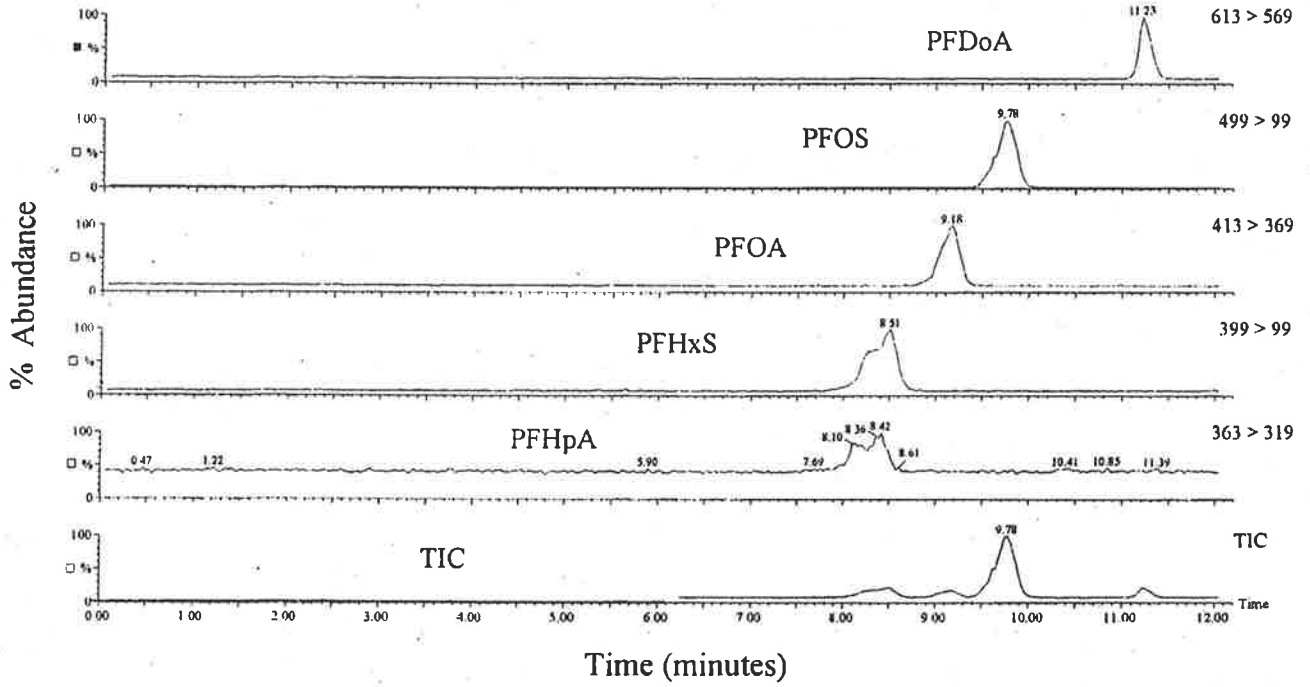

Figure 1. LC/MS/MS chromatogram of perfluorinated compounds, including PFOS, PFHxS, PFOA, PFHpA, and PFDoA (internal standard) in a surface water sample.

(average of 59% ± 20%), and the recoveries for PFOA were 87, 87, and 99% (average of 91% ± 7.6%). Because the percent recoveries for analytes spiked into surface water were similar to those observed in spike and recovery experiments with deionized water, it was presumed that nothing in the sample matrix contributed to a reduction in concentration. For this standard addition experiment, a second 2-mL aliquot of methanol was added to one of the C18 cartridges after the initial 2 mL of methanol was added to elute the analytes of interest. The cartridge was centrifuged, and the second fraction was analyzed by LC/MS/MS in the same manner as the first extract. Interestingly, PFOS was present in the second sample eluate. One approach to improve percent recovery would be to increase the elution volume of methanol from 2 to 5 mL. This needs to be investigated further prior to future analyses of environmental samples to ensure the accuracy of the method.

The RSD calculated from three replicate analyses of two surface water samples, sample 2-4 and sample 3-3, was 4.3 and 6.0% for PFOS, 15 and 8.0% for PFHxS, and 7.5 and 5.3% for PFOA, respectively. In general, the RSD values for replicate analyses for surface water samples indicate good precision within the methodology.

The detection limit of the instrument was defined as those concentrations of PFOS and PFOA that were needed to produce a signal-to-noise (S:N) ratio of 3:1. The instrumental detection limit was 4 and 1 pg for PFOS and PFOA, respectively (Table 1). The limit of quantitation (LOQ) was based upon the lowest calibration curve standard for PFOS and PFOA. The LOQ for a 100-mL surface water sample for PFOS and PFOA was 17 and 9 ng/L, respectively. Previously reported detection limits for PFOS and PFOA in nonconcentrated sera by LC/MS/MS were 1.7 and 1.0 μg/L, respectively.[30]

Optimization of the ^{19}F NMR Method. For the quantitative determination of perfluorinated surfactants, the ^{19}F NMR spectral window was restricted to the range of −55 to −85 ppm. Quantification was based upon the peak area of the terminal CF_3 group (the CF_3 group was the largest peak in each spectrum) and the peak area of the internal standard (TFMAA). Because the chemical shift and coupling constants were approximately the same for the CF_3 groups of PFOS, PFHxS, PFOA, and PFHxA, spectral overlap occurred when quantification was based solely on this substituent. Once the ^{19}F NMR parameters were established for the T_1 relaxation times of the compounds, the response factor from all of the terminal CF_3 groups that were present was the same. Therefore, quantification was based upon calibration curves constructed using a single compound, PFOS, having a relaxation time long enough to allow the relaxation time of all CF_3 groups, thereby allowing complete quantitation of the perfluorinated surfactants present.

Accuracy, Precision, and Detection Limits for ^{19}F NMR. For the ^{19}F NMR methodology, the solid-phase extraction efficiency experiment recoveries of PFOS from deionized water, at 0.15 mg/L and 0.70 mg/L, were 96% ± 18% and 99% ± 10%, respectively, for three replicates at each concentration. The recovery of the mixed standard for a single replicate was 86%, based upon a calibration curve constructed with PFOS. The precision of the ^{19}F NMR method, indicated by the relative standard deviation (RSD), for a single surface water sample (n = 3) was 5.4%. The percent recoveries for spike and recovery experiments indicate that the accuracy of the method is acceptable, and the percent RSD value for a surface water sample performed in triplicate indicates good precision within the ^{19}F NMR methodology.

Table 2. Total Perfluorinated Surfactant Concentration Determined by ^{19}F NMR and LC/MS/MS in Surface Water Samples from an AFFF Spill

sample[a]	sample collection date	^{19}F NMR, *n*	tot concn by ^{19}F NMR[b], $\mu g/L$	tot concn by LC/MS/MS[c], $\mu g/L$
2-1	June 10, 2000	1	<mdl[d]	0.011
2-2	June 10, 2000	1	311	93.5
2-3	June 10, 2000	1	417	114
2-4	June 10, 2000	1	539	133
2-5	June 10, 2000	1	900	185
2-6	June 10, 2000	3	17 000 (5.4%)	2 270
3-1	June 11, 2000	1	<mdl	0.028
3-3	June 11, 2000	1	931	205
3-4	June 11, 2000	1	267	69.3

[a] Sample 2-1 denotes the sample was collected 2 days after the spill (June 10, 2000) at sampling site 1. [b] Total concentration ($\mu g/L$) by ^{19}F NMR represents the concentration determined from the CF_3 chemical shift, approximately −79 ppm. The RSD is given in parentheses for a replicate analysis. [c] Total concentration by LC/MS/MS represents the summation of PFOS, PFHxS, and PFOA concentrations ($\mu g/L$). [d] Method detection limit (mdl) for ^{19}F NMR is 10 $\mu g/L$ for a 100-mL aqueous sample.

Table 3. Individual Perfluorinated Surfactant Concentrations Determined by LC/MS/MS in Surface Water Samples from an AFFF Spill

sample[a]	date collected	n	PFHxS[b] $\mu g/L$	PFOS[b] $\mu g/L$	PFOA[b] $\mu g/L$
2-1	June 10, 2000	1	nd	nd[c]	0.011
2-2	June 10, 2000	1	3.45	89.2	0.81
2-3	June 10, 2000	1	nd	113	0.61
2-4	June 10, 2000	3	5.44 (15%)	126 (4.3%)	1.60 (7.5%)
2-5	June 10, 2000	1	8.22	174	2.49
2-6	June 10, 2000	1	49.6	2210	11.3
3-1	June 11, 2000	1	nd	nd	0.028
3-3	June 11, 2000	3	3.44 (8.0%)	201 (6.0%)	0.513 (5.3%)
3-4	June 11, 2000	1	1.47	66.7	1.14

[a] Sample 2-1 denotes the sample was collected 2 days after the spill (June 10, 2000) at sampling site 1. [b] The relative standard deviation is given in parentheses for replicate analyses. [c] nd: not detected. Method limit of quantitation for a 100-mL sample by LC/MS/MS is 17 and 9 ng/L for PFOS and PFOA, respectively.

On the basis of a signal-to-noise ratio of 3:1, the instrumental detection limit was 0.25 $\mu g/mL$. The detection limit of the method was defined as those concentrations of PFOS that were needed to produce a signal-to-noise of 3:1 and was 10 $\mu g/L$ for a 100-mL surface water sample.

Application to Surface Water Samples. To demonstrate the LC/MS/MS method, nine surface water samples from Etobicoke Creek were analyzed for perfluoroalkanesulfonates and perfluorocarboxylates. Chromatograms obtained in full-scan mode indicated the presence of multiple perfluorinated compounds, all of which had characteristic perfluorocarbon fragmentation.[32,33] One indication of perfluorocarbon fragmentation was that the major daughter ions differed by 50 amu, which corresponds to the mass of CF_2. Additionally, the retention times for the perfluorinated compounds in surface water samples (Figure 1) were coincident to that of the purchased standards.

An unidentified peak having a retention time of 1.6 min was observed in full-scan mode (m/z 50−800). Spectral evidence, including characteristic perfluorocarbon fragmentation, indicated that it may be an amphoteric fluorinated surfactant, such as an amphoteric fluoroalkylamide, which is listed as a proprietary component in some AFFF formulations.[6,34,35] Because the amphoteric fluorinated surfactant component comprises 1.0−5.0% of an AFFF concentrate,[6] this class of fluorinated compounds is potentially present in Etobicoke Creek samples at higher concentrations than the perfluoroalkanesulfonates (0.5 to 1.5% of concentrate composition[6]) and perfluorocarboxylates.

Total perfluorinated concentrations, the summation of PFOS, PFHxS, and PFOA concentrations, in surface water samples ranged from 0.011 to 2270 $\mu g/L$ (Table 2). Perfluorooctanesulfonate was the predominant anionic perfluorinated surfactant detected in surface water samples by this method. The Etobicoke Creek samples had total perfluoroalkanesulfonate concentrations from nondetect (nd) to 2260 $\mu g/L$, with PFOS concentrations ranging from nd to 2210 $\mu g/L$ (Table 3), and generally accounting for >90% of the total perfluoroalkanesulfonate concentrations. Perfluorohexanesulfonate concentrations ranged from nd to 49.6 $\mu g/L$ (Table 3), with the C6 homologue accounting for <10% of the total perfluoroalkanesulfonate concentration. Perfluoroheptanesulfonate and PFBS were observed in surface water samples but were not quantified. This suite of aqueous samples was not monitored for perfluoropentanesulfonate. The observation of a suite of homologues in surface water contaminated with AFFF material is consistent with previous reports of AFFF-contaminated groundwater.[19]

Perfluorooctanoate concentrations ranged from 0.011 to 11.3 $\mu g/L$ (Table 3). Lower chain perfluorocarboxylate homologues, including perfluoroheptanoate (Figure 1), perfluorohexanoate, and perfluoropentanoate were also observed. The finding of perfluorocarboxylate homologues is again in agreement with the observation of perfluorooctanoate and other lower chain perfluorocarboxylate homologues in groundwater contaminated by AFFF.[5,19,24]

An AFFF concentrate[6] was diluted with deionized water, preconcentrated, and analyzed by LC/MS/MS. Perfluorooctanesulfonate, PFHxS, PFOA, and PFHpA were detected in the AFFF concentrate sample and showed retention times and fragmentation patterns similar to the perfluorinated compounds observed in surface water samples collected from Etobicoke Creek and purchased perfluorinated standards. The observation of a suite of homologues for each class of perfluorinated surfactants is reasonable, given that the raw materials used for the syntheses of these compounds are mixtures.[2] Furthermore, the presence of even and odd homologues of perfluoroalkanesulfonates and perfluorocarboxylates in AFFF concentrates and surface water samples is indicative of the electrochemical fluorination manufacturing process.

The second developed method, the ^{19}F NMR method, was employed on the same suite of surface water samples. A typical ^{19}F NMR spectrum (Figure 2) indicates the presence of perfluorinated surfactants in surface water collected from Etobicoke Creek. Total perfluorinated surfactant concentrations ranged from

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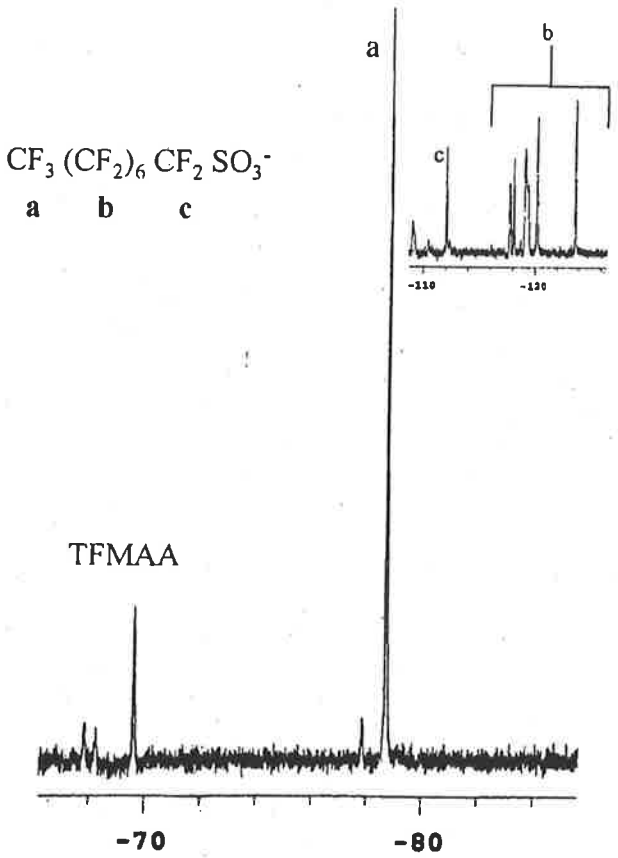

Figure 2. ^{19}F NMR spectrum for a surface water sample highlighting perfluorinated compounds relative to the internal standard TFMAA. The additional peaks from the perfluorocarbon chain are shown in the inset.

the method detection limit (mdl, 10 μg/L) to 17 000 μg/L (Table 2). The creek water samples that were collected upstream of the airport and the spill (samples 2-1 and 3-1) were blank in the region of interest, −79 ppm.

It should be noted that it is unlikely that an additional source of perfluorinated compounds in the ^{19}F NMR spectra originated from agricultural applications: common perfluorinated compounds employed for agriculture purposes include fluometuron, trifluralin, and tefluthrin.[36] The range of ^{19}F NMR chemical shifts for perfluorinated pesticides are generally −60 to −70 ppm, but the chemical shift for perfluorinated surfactants including PFOS, PFHxS, PFOA, and PFHxA was approximately −79 ppm (Figure 2). Additionally, the fluorinated compounds employed for agriculture generally have a single CF_3 group adjacent to an aromatic ring, and therefore, the CF_3 peak is a singlet. In contrast, the CF_3 peaks that were observed for perfluorinated surfactants were as multiplets.

As can be observed through the comparison of the results (Table 2), discrepancies exist between the total perfluorinated surfactant concentrations measured by the two independent methods. With each surface water sample, there was considerable variance between the total perfluorinated surfactant concentration determined by ^{19}F NMR and the total concentration (the summation of PFOS, PFHxS, and PFOA concentrations) by LC/MS/MS. The total concentrations for sample 2-6, 17 000 and 2270 μg/L for ^{19}F NMR and LC/MS/MS, respectively, illustrate the differences. These discrepancies may be attributed to the presence of other surfactants in the surface water samples that yield a ^{19}F NMR spectrum similar to that of perfluoroalkanesulfonates and perfluorocarboxylates, therefore, contributing to the peak area at −79 ppm and yielding higher total concentrations. One example of a surfactant potentially present in the NMR solution would be an amphoteric fluorinated surfactant having a CF_3 group that contributes to the ^{19}F NMR spectra. This is a plausible explanation, given the mass spectral observations by liquid chromatography/mass spectrometry in full-scan mode.

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CONCLUSIONS

Two independent analytical techniques were developed to determine the concentration of perfluorinated surfactants, including perfluoroalkanesulfonates and perfluorocarboxylates, in aqueous samples. Results from both methodologies indicate perfluorinated surfactants were directly released into the environment at mg/L levels. Negative electrospray ionization LC/MS/MS is a powerful tool to characterize and determine concentrations of a range of perfluorinated surfactants in environmental matrixes. Employing LC/MS/MS allowed for the unambiguous determination of individual perfluorinated surfactant compounds in Etobicoke Creek samples after a fire-fighting foam spill. The ^{19}F NMR method (with its specificity to fluorine) complements the LC/MS/MS methodology by providing unequivocal structural information. This structural information will have future applications in the study of linear and branched isomers of perfluorinated surfactants. Additionally, when high concentrations of the analytes are present, as was the case in the surface water collected immediately following the AFFF spill, the ^{19}F NMR can give valuable information in a short period of time. When combined, the developed methods permit the characterization of surface water samples for the presence of several classes of fluorinated surfactants. Our current research focuses on employing the developed LC/MS/MS and ^{19}F NMR methodologies for the study of the persistence of perfluorinated surfactants in Etobicoke Creek aqueous and biota samples.

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